Vol. 113, No. 3 December, 1957

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

CHEMICAL ANALYSES OF ANTERIOR AND POSTERIOR BLASTOMERES OF CIONA INTESTINALIS

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Segregations of specific cytoplasms occur during early cleavage of the ascidian egg (Conklin, 1905) of which the most obvious is the localization of the myoplasm, presumptive for larval musculature, in the posterior cells at the four-cell stage. Cytological (Meves, 1913; Duesberg, 1915; Conklin, 1931) and cytochemical (Ries, 1937) studies indicate the localization of granules, presumably mitochondria, within the myoplasm. Recently Reverberi (1956) followed the distribution of mitochondria during development, using the vital stain, Janus green. The quantitative measurements of cytochrome oxidase in anterior and posterior blastomeres (Berg, 1956) gave a biochemical confirmation of the above studies as regards the localization of mitochondria.

The present study is a continuation of quantitative chemical analyses of anterior and posterior blastomeres from the four-cell stage of *Ciona*, choosing constituents which might be expected to be located on cellular particles. The minute amounts of cytoplasm available require the use of microchemical methods and limit the number of substances which may be studied; however, differences in activities of succinic dehydrogenase, apyrase, acid phosphatase, and ribonucleic acid have been found in homogenates of the two types of cells.

Methods

Chorions were digested off unfertilized *Ciona* eggs in 3 per cent protease in sea water. The "naked" eggs were washed thoroughly, fertilized, and transferred to agar-coated dishes. At the end of the first cleavage the blastomeres were separated in large numbers by agitation and as each of these cleaved in turn, the anterior and posterior cells were separated with the tip of a fine braking pipette and segregated into different dishes.

The posterior blastomere is recognizable by an elongated shape and a clear cytoplasmic cap (Castle, 1896). These characteristics are transitory and the period for separation is critical; separation before completion of the cleavage furrow results in cytolysis whereas shortly after cleavage identification becomes increasingly difficult due to sphering of the posterior cell. This period was extended by lowering the temperature to 8–10° C. after completion of cleavage, thus prolonging the elongated state of the posterior cell. Furthermore it was discovered that with oblique

lighting the posterior cell, even after sphering, exhibits a bright crescentic rim of cytoplasm which is the remnant of the clear cytoplasmic cap. This persists for some minutes after cleavage and greatly extends the length of time during which identification is possible.

Separation of blastomeres was begun as soon as possible after cleavage, using the difference in shapes for identification. As the posterior cells began to round up, an oblique lighting was adopted and separation continued, using the bright rim of the posterior cell as a marker. In this manner several hundred blastomeres could be isolated, a considerable improvement over initial attempts where a maximum of 30–40 were obtained (Berg, 1956).

The desired number of segregated blastomeres was counted and transferred to 0.1-ml. centrifuge tubes by means of a braking pipette. After light centrifugation excess sea water was removed, a few μl. of homogenizing solution were added and the cells homogenized by drawing them in and out of a fine-bore pipette. The homogenization was carried out at 1–2° C., attained by placing the centrifuge tube in a previously chilled copper block. All micromethods were spectrophotometric, using the Beckman spectrophotometer adapted for the use of microcuvettes (Lowry and Bessey, 1946).

Succinic dehydrogenase activity in homogenates was measured by the method of Cooperstein *et al.* (1950). The blastomeres were homogenized in 1 μl. of 0.19 *M* sodium succinate; 5 μl. of 0.19 *M* succinate and 2 μl. of 0.03 *M* sodium cyanide were added and the mixture transferred to 45 μl. of 2×10^{-5} cytochrome *c* contained in a microcuvette. All solutions were buffered with 0.04 *M* phosphate buffer at pH 7.4. The reduction of cytochrome *c* was followed at 550 mμ for three minutes at the end of which time a few grains of sodium hydrosulfite were added to completely reduce the cytochrome *c*. A semi-logarithmic plot of the readings, after substraction of the optical density of reduced cytochrome *c*, gave a straight line from which a velocity constant, (Δlog ferricytochrome $c/\Delta T$), could be calculated. Succinic dehydrogenase activities were expressed as velocity constants for rates of reduction of cytochrome *c*.

Acid and alkaline phosphatases and apyrase were measured by micromethods described by Lowry *et al.* (1954). For acid phosphatase 10 μl. of substrate (8 mM disodium p-nitrophenyl phosphate in 0.05 *M* succinate buffer, pH 5) were mixed with the homogenate. After thirty minutes of incubation at 25° C., 45 μl. of 0.1 N NaOH were added, with immediate mixing, and read at 410 mμ within thirty minutes.

Alkaline phosphatase was measured in a similar manner except that a buffer (2 amino-2 methyl-1-propanol) at pH 10 was used with an incubation period of one hour. It was not necessary to carry out protein precipitations in determinations of either acid or alkaline phosphatase. Blanks were prepared by separate incubation of substrate and homogenate with mixing just before addition of NaOH.

Apyrase measurements were made by homogenizing the cells in 0.75 per cent sodium desoxycholate, a procedure which considerably increases the enzyme activity presumably due to particle breakdown. Ten μl. of substrate (2.5 mM adenosinetriphosphate in 0.05 *M* tris hydroxy-amino methane at pH 8.0) were added to the homogenate and the mixture incubated for one hour at 25° C. Protein was precipitated by adding 2 μl. of 30 per cent trichloracetic acid and, after centrifugation, the supernatant was transferred to another tube with 100 μl. of molybdate-ascorbic

acid reagent and read at 870 mμ. Blanks were prepared by separate incubation of substrate and homogenate with mixing at the time of addition of trichloracetic acid. Both apyrase and acid phosphatase activities were expressed as optical densities after subtraction of blank values.

Ribonucleic acid was determined by a micromethod based on the procedure of Ogur and Rosen (1950). Cells were extracted with 60 μl. of cold 70 per cent alcohol for 5–10 minutes followed by extraction with 60 μl. of warm alcohol-ether (3:1). After a few minutes extraction with cold 0.1 *M* perchloric acid, a final extraction with 45 μl. of 1.0 *M* perchloric acid was carried out for 48 hours.

A typical absorption curve for ribonucleic acid was obtained with the latter extract and the optical density at 260 mμ was used as a measure of the amount. Repeated test extractions with 70 per cent alcohol, alcohol-ether, and 0.1 *M* perchloric acid demonstrated that these solutions removed, within a few minutes, all amino acids, polypeptides and acid-soluble substances which absorb at 260 mμ. Although most extractions were carried out for a longer time, nearly all the ribonucleic acid was removed within 24 hours.

Protein was measured by the method of Lowry *et al.* (1951). The cells were placed in a 0.5-ml. test tube and 100 μl. of alkaline copper solution added and mixed. After 10 minutes, 10 μl. of diluted Folin reagent were added with immediate mixing and the sample read at 750 mμ. Addition of the Folin reagent is critical and the reliability of color development depends upon the rapidity and effectiveness of the mixing. On a micro scale this is difficult to control and the resulting variability seriously limits this method.

Calibration curves (Fig. 1) were made for each of the micromethods. Fertilized eggs, with the chorions removed, were used for these tests and as shown in Figure 1 the optical density measurements, or velocity constants for succinic dehydrogenase, are proportional to enzyme activities as represented by the number of eggs. The optical densities at 260 mμ of perchloric acid extracts, representing amounts of ribonucleic acid, are proportional to the number of eggs extracted.

Results

Anterior and posterior blastomeres of *Ciona* were separated and kept at 1° C. in agar-coated dishes until ready for counting and transference to the reaction tubes. From the calibration curves (Fig. 1) an estimation was made of the number of blastomeres necessary in each test for reliable measurements of the constituent. Thirty to forty cells in each tube were sufficient for enzyme and protein measurements, whereas nearly one hundred were required for a reliable measure of ribonucleic acid.

All determinations of enzymes, ribonucleic acid, and proteins in anterior and posterior blastomeres were paired; *i.e.*, two reaction tubes were used, one containing anterior cells, the other containing an equal number of posterior cells. The analyses were then carried out simultaneously. Due to the variability of results obtained with microchemical methods it was necessary to repeat the analyses a number of times and, as each experiment was on eggs from different animals and carried out under slightly different conditions, the data are expressed as ratios of activities or concentrations for each paired experiment. The average of these ratios is used to summarize the results although statistical significance was calculated directly from the paired series.

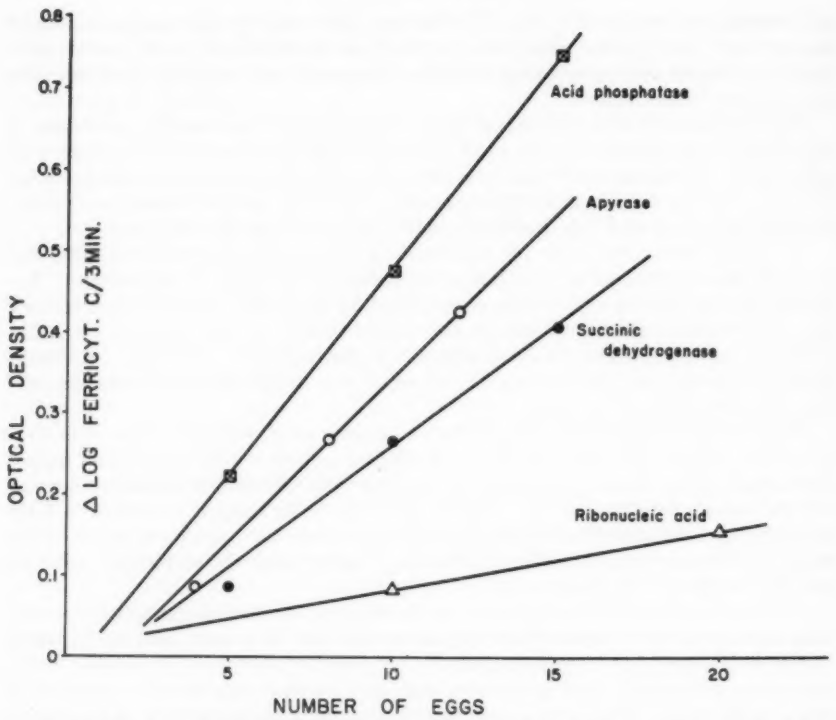

FIGURE 1. Acid phosphatase (activity expressed as optical density), apyrase (optical density), succinic dehydrogenase (Δ log ferricytochrome *c*/3 minutes) and ribonucleic acid (optical density) in homogenates of *Ciona* eggs.

The data for eighteen paired measurements of acid phosphatase in anterior and posterior cells are summarized in Table I. The anterior cells contain 12 per cent more of this enzyme than posterior cells, a difference which is significant at the one per cent level. A number of these experiments were carried out using sodium desoxycholate in the homogenization medium, with no detectable increase in enzyme activity.

TABLE I

Acid phosphatase, expressed as optical densities, in homogenates of anterior and posterior cells of Ciona

Number of tests	Acid phosphatase in anterior cells	Acid phosphatase in posterior cells	Average ratio of paired determinations (ant./post.)
18 (25–40 cells for each test)	0.318	0.287	1.12 ± 0.03

A homogenate of thirty or more *Ciona* eggs was necessary to obtain even a detectable activity of alkaline phosphatase and accordingly it was not feasible to measure the activity of this enzyme in isolated blastomeres. The very low alkaline phosphatase activity in early cleavage stages is also characteristic of the mollusk *Mytilus edulis* and the sea urchin *Strongylocentrotus purpuratus*.

TABLE II

Succinic dehydrogenase, expressed as velocity constants for reduction of cytochrome c, in homogenates of anterior and posterior blastomeres

Number of cells	Succinic dehydrogenase in anterior cells	Succinic dehydrogenase in posterior cells	Ratio (ant./post.)
45	0.048	0.071	0.68
40	.033	.062	.53
45	.025	.070	.36
40	.043	.071	.61
45	.042	.089	.47
		Average	0.53 ± 0.05

As would be expected on the basis of earlier measurements of cytochrome oxidase (Berg, 1956), succinic dehydrogenase activity is greater in posterior cells. The average of five experiments shows that homogenates of posterior cells contain twice as much of this enzyme as anterior cells (Table II).

TABLE III

Apyrase, expressed as optical densities, in homogenates of anterior and posterior blastomeres

Number of blastomeres	Apyrase in anterior cells	Apyrase in posterior cells	Ratio (ant./post.)
44	0.286	0.342	0.84
66	.334	.451	.74
50	.164	.260	.63
60	.230	.352	.65
30	.229	.342	.67
30	.269	.328	.82
30	.269	.412	.65
30	.169	.240	.70
40	.282	.471	.60
		Average	0.70 ± 0.03

Apyrase measurements, summarized in Table III, show that the activity of this enzyme in homogenates of anterior cells is 70 per cent that in homogenates of posterior cells. In adult tissues (Frank *et al.*, 1950) and in amphibian embryos (Barth and Jaeger, 1947) apyrases with different pH activity characteristics have been extracted and thus for quantitative measurements of apyrases from different sources

it is necessary to determine pH-activity curves. Accordingly an attempt was made to determine the effect of pH on apyrases from anterior and posterior cells.

Succinate, tris-maleate, and ammediol buffers were used to cover the pH range from 5 to 9.3; pH values were checked with a glass electrode on mixtures prepared on a macroscale exactly as used for microanalyses. Apyrase activities were determined simultaneously at four different pH's in a paired series with thirty anterior or posterior blastomeres in each reaction tube. It was not feasible to cover the entire pH range in any one test; accordingly it was necessary to overlap pH values in

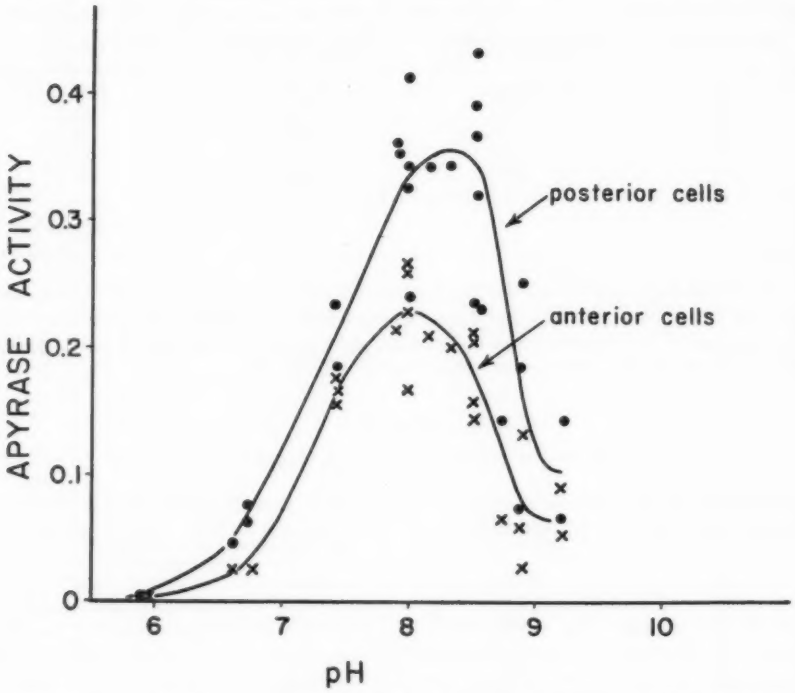

FIGURE 2. Apyrases in homogenates of anterior and posterior blastomeres; pH activity curves.

different experiments. The considerable variability of determinations was probably in part due to the necessity of constructing the curves from data obtained on different batches of eggs.

The pH-activity curves of apyrases from anterior and posterior cells (Fig. 2) are very similar although the optimum for posterior cell apyrase seems more alkaline. The difficulties of obtaining a pH-activity curve on such minute amounts of enzyme are so considerable that this slight difference cannot be considered significant.

The data for paired ribonucleic acid extractions, listed in Table IV, indicate that anterior cells contain 9 per cent less ribonucleic acid than posterior cells, a difference

which is significant. This was at first thought to be due to a slight volume difference between anterior and posterior cells. Visual observation of the four-cell stage often gives the impression that the posterior cells are larger. However, careful measurements of volumes by different methods failed to show any significant difference in size.

Diameters of blastomeres, with subsequent cleavages prevented by KCN or fixation in trichloracetic acid, were measured with an ocular micrometer. The average volume of 85 anterior blastomeres, as calculated from diameter measurements with cleavage inhibited by 3×10^{-4} *M* KCN, was 97 per cent that of 75 posterior cells. The average volume of 134 anterior cells, after fixation in 2 per cent trichloracetic acid, was 98 per cent that of 178 posterior cells. Although with both types of measurements the average volume of the anterior cells was slightly less, statistical analyses of the data failed to show significant differences of these values.

TABLE IV

Ribonucleic acid, expressed as optical densities at 260 mμ, extracted from anterior and posterior blastomeres

Number of blastomeres	RNA in anterior cells	RNA in posterior cells	Ratio (ant./post.)
130	0.285	0.308	0.93
140	.230	.272	.85
114	.216	.240	.90
94	.202	.234	.86
112	.302	.301	1.00
64	.160	.170	.94
70	.154	.200	.77
130	.268	.295	.91
75	.221	.215	1.03
100	.140	.146	.96
120	.271	.320	.85
100	.237	.252	.94
		Average	0.91 ± 0.02

An additional determination of volumes was made indirectly by measuring relative amounts of protein in the two types of cells using a micromethod (Lowry *et al.*, 1951). Twenty-four paired measurements of protein, using 15–40 cells in each test, gave an average protein ratio (ant./post.) of 0.99 ± 0.046. The difference in ribonucleic acid content thus cannot be due to a volume difference and must represent a slight localization of this constituent.

DISCUSSION

A localization of indophenol blue oxidase, presumably cytochrome oxidase, and succinic dehydrogenase in the ascidian embryo has been demonstrated by cytochemical methods (Ries, 1937; Reverberi and Pitotti, 1939; Mancuso, 1952). The microchemical measurements of cytochrome oxidase (Berg, 1956) and those of succinic dehydrogenase in the present study confirm the above cytochemical observations as to the localization of these enzymes in an early cleavage stage.

Information on the intracellular localization of enzymes in the ascidian egg is lacking; however a possible interpretation of the distribution of cytochrome oxidase, succinic dehydrogenase, and apyrase is that they are localized in mitochondria which are unequally distributed in the early cleavage blastomeres. There is considerable evidence that these enzymes are intracellularly located in mitochondria of a variety of cells (reviewed by Schneider and Hogeboom, 1951, 1956). A localization of mitochondria in the myoplasm of the ascidian egg has been demonstrated by cytological methods (Meves, 1913; Duesberg, 1915) and more recently by vital staining with Janus green (Reverberi, 1956). Reverberi (1957a) also presents evidence that cytochrome oxidase is intracellularly localized in the Janus green-staining mitochondria.

It is doubtful that the granules described by Duesberg (1915) and Conklin (1931), classified by them as mitochondria according to morphological and staining criteria, are responsible for the unequal distribution of enzymes. The granules in *Ciona* and "yellow mitochondria" in *Styela* are displaced by centrifugation to the centripetal pole (Conklin, 1931) whereas in centrifuged eggs the oxidase reactions are lacking in this region (Ries, 1939). The yellow granules in *Styela* may be displaced by light centrifugation without altering the localization of the oxidase reactions (Ries, 1942). In centrifuged homogenates of *Ciona* eggs cytochrome oxidase is found in the heavier fraction (Berg, 1956). Furthermore Reverberi (1957a) describes rod-like mitochondria, stainable with Janus green, which, in the centrifuged egg, collect at a different location than the granules described by Duesberg and Conklin. It appears probable, as Reverberi (1957a) also suggests, that several types of mitochondria are localized in the myoplasm of the ascidian embryo.

The intracellular localization of apyrase is probably to some extent within mitochondria, as has been shown for other types of cells (Schneider and Hogeboom, 1951, 1956), and the higher apyrase content of posterior cells most likely results from the segregation of mitochondria into these cells. The average ratio of activities in the two types of cells differs significantly from those for cytochrome oxidase or succinic dehydrogenase, which is interpreted as due to a more heterogeneous intracellular localization of this enzyme as compared to the oxidative enzymes.

Barth and Jaeger (1947) demonstrated that apyrases with different pH activity curves are present in several protein fractions of the amphibian embryo. A similar fractionation of proteins and measurements of the associated apyrases were not possible on *Ciona* blastomeres, due to the minute amounts of material available for analyses. The measurements made therefore represent total apyrase activities of the homogenates and, although there may be qualitatively different apyrases, the similarity of the pH-activity curves for anterior and posterior blastomeres indicates that segregation of this enzyme at the second cleavage is mainly quantitative.

Although acid phosphatase has been found to be intracellularly localized in small mitochondria of adult cells (Appelmans *et al.*, 1955), in the present experiments the opposite distribution of oxidative enzymes and acid phosphatase suggests a non-mitochondrial localization of the latter. It is possible that the localization of acid phosphatase may be a consequence of an unequal distribution of mitochondria. Thus a 2:1 distribution of mitochondria, as indicated by enzyme analyses, might cause displacement of a non-mitochondrial constituent into anterior cells.

Without information on the intracellular localization of ribonucleic acid in the ascidian egg, little can be said regarding the higher ribonucleic acid content of the

posterior cells. A factor other than mitochondrial segregation may be involved since mitochondria have a low content of ribonucleic acid (Schneider and Hogeboom, 1951, 1956).

The results are not due to volume differences since extensive measurements, discussed previously, of diameters and total protein in the two types of cells failed to demonstrate any significant differences in volumes. A differential solubility of ribonucleic acid in anterior and posterior blastomeres might lead to erroneous results; however, there is no indication of this, in that continuous extraction for four days with perchloric acid did not change the ratio of amounts extracted.

These quantitative analyses do not, of course, give any information as to the significance of the chemical differences in subsequent differentiation. A few preliminary experiments of rearing embryos in graded concentrations of KCN failed to show, by visual observation of whole embryos, any obvious differential effects on differentiation of anterior and posterior cells. Recently, however, Reverberi (1957b) has shown specific effects of sodium azide, malonate, and selenite on differentiation of the musculature of ascidian larvae, an effect presumably due to blocking activities of mitochondrial enzymes. Previously Ries (1939), by displacement of cytoplasmic areas with centrifugation, had concluded that the presence of the oxidative enzymes was essential for muscle differentiation.

Although localization of oxidases may be of significance in subsequent differentiation of ascidian embryos and several other mosaic forms (*Tubifex*, Lehmann and Wahli, 1954; *Nereis*, Reverberi and Pitotti, 1940; *Myzostoma*, Pitotti, 1947), this is not a common process during cleavage of all mosaic eggs.

Quantitative measurements of cytochrome oxidase in AB and CD blastomeres of *Mytilus edulis* indicated no unequal distribution of the enzyme in this mosaic egg (Berg, unpublished). First-cleavage blastomeres were isolated by previously described methods (Berg, 1950) and cytochrome oxidase measured by a microspectrophotometric method (Berg, 1956). Twelve paired measurements of the enzyme in homogenates of isolated blastomeres gave an average ratio of enzyme activity for the two types of cells (AB/CD) of 0.98 ± 0.03 after correction for volume differences.

Furthermore cytochemical tests for oxidative enzymes failed to reveal segregation of these enzymes during early cleavage of *Sabellaria* (Raven *et al.*, 1950), *Chaetopterus* and *Pomatocerus* (Ries, 1937), *Hydroides* (Reverberi and Pitotti, 1940) and *Limnaea* (Raven, 1946).

I am indebted to Professor Martin W. Johnson of the Scripps Institution of Oceanography, University of California, La Jolla for generously providing space and facilities in his laboratory where a portion of this work was carried out.

Summary

Anterior and posterior blastomeres of the four-cell stage of *Ciona* were separated for quantitative microchemical analyses of succinic dehydrogenase, apyrase, acid and alkaline phosphatases, and ribonucleic acid. Larger amounts of succinic dehydrogenase, apyrase and ribonucleic acid were found in homogenates of posterior cells whereas acid phosphatase activity was higher in anterior cells. The pH-activity curves of apyrases from anterior and posterior cells are similar, indicating a quantitative segregation of this enzyme. The unequal distribution of succinic dehydrogenase, apyrase and, possibly indirectly, acid phosphatase, is probably the result of a segregation of mitochondria.

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THE EFFECTS OF SOME DEVELOPMENTAL INHIBITORS ON THE PHOSPHORUS BALANCE OF AMPHIBIAN GASTRULAE

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Considering the available evidence, it is possible that the morphogenetic movements of gastrulating amphibian embryos are energetically coupled to exergonic metabolic processes, and it is reasonable to assume that the coupling is mediated by energy-rich phosphate-bonds. On this basis, it is possible to propose explanations for the well-known inhibitory effects upon gastrular development of such agents as anaerobiosis, azide and dinitrophenol (Ornstein and Gregg, 1952; Gregg and Ornstein, 1953), for all of these are believed to dissever or restrict energetic couplings: anaerobiosis by switching out the aerobic exergonic processes of the Krebs cycle, azide perhaps by promoting the immediate remineralization of newly esterified phosphorus in the Embden-Meyerhof system (Spiegelman, Kamen and Sussman, 1948), and dinitrophenol perhaps by promoting the catalytic remineralization of esterified phosphorus by mitochondrial dephosphorylases (Hunter, 1951) or by direct "quenching" of energy-rich phosphate bonds (Middlebrook and Szent-Györgyi, 1955). If the energetic demands of gastrular movements are at all appreciable, then embryos treated with such agents might be expected to exhibit decreases in their stores of esterified phosphorus, accompanied by corresponding increases of their inorganic phosphorus contents. The experiments reported in the sequel are intended to test this proposal.

Methods

Obtaining and rearing embryos. Fertilized eggs were obtained by stripping eggs from gravid *Rana pipiens* females into suspensions of active sperm (*R. pipiens* or *R. sylvatica*). After about two hours, the clutches of embryos were cut with scissors into small groups, dispersed thinly among several finger bowls, and reared at a temperature of 14–15° C. until required for use. The medium in the bowls, changed daily, was 10% amphibian Ringer's solution without phosphate or bicarbonate. Just before use, the embryos were freed of their jelly-coats with forceps. Their developmental stages were determined by reference to the tables of Shumway (1940).

Treatment with inhibitors. Solutions of sodium azide and 2, 4 dinitrophenol were prepared by dissolving weighed samples in aliquots of the same medium in which embryos were reared. The treatment consisted in placing 25 or 30 Stage 10 embryos in a covered stender dish containing 5 or 10 ml. of inhibitor solution for 24 hours at 14–15° C. At the end of this period their developmental stages were noted, then they were washed rapidly with distilled water and lyophilized (see below). Afterward, they were dry-stored in the freezing compartment of a refrigerator until phosphorus analyses could be made, usually within two or three days.

[1] This investigation was supported in part by a research grant, No. A-1082, from the Public Health Service.

Anaerobiosis. For each experiment, 25 or 30 Stage 10 embryos were put into an Erlenmeyer flask fitted with a two-hole rubber stopper carrying a gas-inlet tube dipping into the medium in the flask (50 ml. of 10% amphibian Ringer's solution, without phosphate or bicarbonate) and a gas-outlet tube. Nitrogen alone, or 95% N_2 : 5% CO_2 (both previously deoxygenated over hot copper), or hydrogen, was then bubbled through the flasks for one hour, after which the inlet and outlet tubes were closed off with pinch clamps. Controls were prepared similarly, except that air, instead of nitrogen or hydrogen, was bubbled through the medium. After 24 hours (at 14–15° C.), the embryos were removed from the flasks, quickly staged, washed in distilled water and lyophilized. These steps preliminary to freeze-drying were carried out as rapidly as possible to prevent the occurrence of aerobic recovery processes. As before, frozen-dried embryos were stored for a short time, if necessary, in the freezing compartment of a refrigerator.

(The authors are grateful for extensive assistance from Dr. Sasha Malamed in this part of the work.)

Lyophilizing. Embryos were dried *in vacuo* in the frozen state with the help of an all-metal apparatus of conventional design built by Mr. Andrew Pfeiffer, Old Lyme, Connecticut. Washed embryos were placed with a minimum amount of distilled water in 10 × 75 mm. Pyrex test tubes which were then partly immersed in a Cellosolve-dry ice slush. After one or two minutes, the tubes were transferred to the drying apparatus. Drying was usually complete in three hours. Samples for chemical analysis were weighed out as rapidly as possible in a closed balance containing a silica-gel desiccant wafer, since lyophilized amphibian embryos are quite hygroscopic.

Phosphorus analyses. Total phosphorus (P_T), total acid-soluble organic phosphorus (P_{AO}) and inorganic phosphorus (P_I) were estimated, in micro- and semimicro amounts, using the methods of Lowry *et al.* (1954). Since we made no essential departures from their recommendations, the reader is referred to their paper for procedural details. The results of analyses are expressed as micrograms P per milligram dry weight of embryo.

Discussion of Results

Azide and dinitrophenol. At a temperature of 14–15° C., normal *R. pipiens* embryos will develop from Stage 10 (early gastrula) to Stage 12 (late gastrula) in about 24 hours. In the presence of inhibitors, morphogenesis may be reduced in amount or abolished altogether, with varying degrees of recovery following the cessation of inhibitory treatment (Table I).

After 24 hours in 10^{-5} *M* azide, *R. pipiens* embryos immersed at Stage 10 will have reached early Stage 12, with no observable after-effects. Embryos similarly treated with 10^{-4} to 10^{-3} *M* azide will have gastrulated only partially, with after-effects ranging from slight to severe. In 10^{-2} *M* azide, no gastrulation occurs, and the after-effects are very severe.

After 24 hours in 10^{-7} *M* 2, 4 dinitrophenol, *R. pipiens* embryos immersed at Stage 10 exhibit no developmental peculiarities, and there are no detectable after-effects of this treatment. In 10^{-6} to 10^{-5} *M* dinitrophenol development is usually retarded in various degrees and the after-effects range from moderately severe to complete failure of recovery. Dinitrophenol in concentrations of 10^{-4} *M*, or greater, inhibits gastrulation altogether, and no recovery has yet been observed.

TABLE I

Development of R. pipiens embryos after treatment with azide or dinitrophenol, 14°–15° C. Stage 10 jelly-free embryos were immersed in inhibitors for 24 hours, then washed daily in 10% amphibian Ringer's solution until controls reached Stage 21. S_{C12} = stage of treated embryos after 24 hours when controls are in Stage 12. S_{C21} = stage of treated embryos when controls are in Stage 21

Inhibitor	Molar concentration	S_{C12}	S_{C21}	Morphological condition
Azide	10^{-5}	12^-	21	Normal
	10^{-4}	$10\frac{1}{2}$–12	20	Mostly normal. Gills underdeveloped but with circulation
	10^{-3}	10–11	18–20	Thickened tail buds, swellings on flanks, underdeveloped gills with circulation
	10^{-2}	10	18–19	Large yolk plugs, spina bifida, swellings on flanks, muscular response
2,4 Dinitrophenol	10^{-7}	12	21	Normal
	10^{-6}	11–12	11–18	Most in Stage 17–18, fairly normal, some with spina bifida
	10^{-5}	10^+–11^-	10^+–16	Most in Stage 10^+–11^-. Others misshapen, with large yolk plugs, whitish "bloom" on surface coat
	10^{-4}	10	10	"Bloom"
	10^{-3}	10	10	"Bloom"

Thus, it would seem that embryos which have been *totally* blocked with azide can afterwards attain a considerable degree of morphological maturity, something which is apparently denied embryos similarly blocked with dinitrophenol. Just possibly, however, this difference between azide and dinitrophenol may be less related to specific differences in their chemical activities than to differences in their separate abilities to pass outwards through the vitelline membrane. For, we found that even repeated washing fails to remove all of the dinitrophenol from treated embryos; part of it, at least, remains visibly concentrated in the perivitelline fluid. Whether there is a similar retention of azide we do not know, since solutions of this inhibitor are colorless.

We turn now to discuss the effects of such treatments upon the phosphorus balance of amphibian gastrulae.

TABLE II

Developmental stage and phosphorus balance of R. pipiens gastrulae after 24 hours exposure to sodium azide, 14–15° C. For meanings of column headings, see section on Methods. Numerals following ± designate standard deviations. Parenthesized numerals designate numbers of experiments upon which values are based

Molar conc. azide	Shumway stage	P_T	P_{AO}	P_I
0	10	13.0 ± 0.4 (3)	0.59 ± 0.09 (3)	0.12 ± 0.02 (3)
0	12–12^+	12.9 ± 0.7 (3)	0.59 ± 0.11 (3)	0.13 ± 0.02 (3)
10^{-5}	12^-	13.0 ± 0.3 (2)	0.65 (1)	0.16 ± 0.03 (2)
10^{-4}	$10\frac{1}{2}$	12.8 ± 0.5 (3)	0.43 ± 0.02 (2)	0.23 ± 0.02 (3)
10^{-3}	10^+	13.1 ± 0.4 (2)	0.44 ± 0.04 (2)	0.29 ± 0.05 (2)
10^{-2}	10	13.2 ± 0.5 (2)	0.44 ± 0.01 (2)	0.30 ± 0.01 (2)

Table II shows that the total phosphorus content of *R. pipiens* gastrulae, exposed for 24 hours to 10^{-5} to 10^{-2} *M* azide, is the same as that of untreated control gastrulae. This is an important point because (together with the data in Table I) it suggests that such embryos are practically undamaged, otherwise a certain amount of leakage of phosphorus might be expected. The other two phosphorus fractions, however, exhibit changes, for as the concentration of environmental azide is increased, there is an increase of the inorganic phosphorus content of treated embryos, accompanied by a quantitatively similar decrease in the level of acid-soluble organic phosphorus. Furthermore, as the concentration of environmental azide is progressively elevated, there is a parallel increase in the severity of gastrular retardation.

The data summarized in Table III show that a similar set of results may be obtained with 2, 4 dinitrophenol at concentrations of 10^{-5} *M*, or lower. At concentrations greater than 10^{-5} *M*, however, gastrulae are damaged to such an extent that they begin to leak phosphorus; this is clearly shown by a correlated decline in the levels of all three phosphorus fractions.

Finally, it should be noted that the gastrulation of untreated control embryos is unaccompanied by any significant alterations of phosphorus balance.

TABLE III

Developmental stage and phosphorus balance of R. pipiens gastrulae after 24 hours exposure to 2,4 dinitrophenol (DNP), 14–15° C. For meanings of column headings, see section on Methods. Numerals following ± designate standard deviations

Molar conc. DNP	Shumway stage	P_T	P_{AO}	P_I	No. expts.
0	10^-–10	13.1 ± 0.7	0.59 ± 0.07	0.13 ± 0.01	2
0	12	13.1 ± 0.1	0.58 ± 0.04	0.13 ± 0.01	2
10^{-6}	12	12.9 ± 0.1	0.60 ± 0.09	0.17 ± 0.05	2
10^{-5}	10^+–11^-	12.6 ± 0.2	0.45 ± 0.01	0.31 ± 0.02	2
5×10^{-5}	10^-	11.9	0.34	0.22	1
10^{-4}	10^-–10	12.4 ± 0.3	0.19 ± 0.09	0.08 ± 0.08	2

Those results suggest the following interpretation. If the movements of gastrulation of normal untreated embryos demand an available supply of phosphate-bond energy, the resulting draughts upon esterified phosphorus are immediately reimbursed, and the phosphorus balance is steadily maintained. This result is in full agreement with the data of Barth and Jaeger (1947), summarized in their Table 1. It is also consistent with the view that gastrulation is a complex of morphogenetic movements whose execution requires no expenditure of energy. The results with azide and dinitrophenol suggest the contrary, however, because of the correlation between the presence of these inhibitors, the reduction of esterified phosphorus, the elevation of inorganic phosphorus, and the retardation of gastrular movements. For it is difficult to explain this correlation except by assuming that in the presence of inhibitors the production of esterified phosphorus is uncoupled from its utilization as a source of morphogenetic energy because it is made available to enzymes catalyzing its remineralization (see the remarks at the beginning of this paper). Of the complex of movements, Gregg and Ornstein (1953) have presented evidence suggesting that epiboly is the most sensitive to treatment with dinitrophenol, while

TABLE IV

Developmental stage and phosphorus balance of R. pipiens gastrulae after 24 hours anaerobiosis, 14°–15° C. For meanings of column headings, see section on Methods. Numerals following ± designate standard deviations. P values in A and B are listed separately in order that the former may be compared with those obtained from hybrid embryos prepared from the same five clutches of eggs (Table V)

	Treatment	Shumway stage	P_T	P_{AO}	P_I	No. expts.
	Control	10	13.5 ± 0.4	0.58 ± 0.04	0.13 ± 0.02	5
A	Control	12	13.2 ± 1.0	0.55 ± 0.03	0.13 ± 0.01	5
	Nitrogen	11	13.5 ± 1.1	0.56 ± 0.05	0.15 ± 0.01	5
	Control	10^+	12.4	0.58	0.12	1
B	Control	12	13.00	0.52	0.13	1
	Nitrogen	11	12.9	0.52	0.18	1
	Control	10	12.4	0.58	0.13	1
C	Control	12^-	12.5	0.60	0.13	1
	Hydrogen	11^-	12.5	0.64	0.18	1

epiboly and notochordal elongation are most affected by the presence of azide; but the exact relations of their results to the present ones are yet to be worked out.

Anaerobiosis. Table IV summarizes the results of experiments designed to show the effects of 24 hours anaerobiosis on the phosphorus balance of *R. pipiens* gastrulae. Embryos are morphologically retarded under these conditions, but, in general, there is no alteration of the phosphorus balance other than a slight elevation of the inorganic phosphorus level. The total phosphorus and acid-soluble organic phosphorus levels are unaffected. There is thus a considerable morphogenetic effect of anaerobiosis, apparently unaccompanied by a decrease in the stored phosphate-bond energy potentially available. This conclusion is not in agreement with that of Barth and Jaeger, who found that anaerobioses of 10 to 22 hours duration resulted in a considerable decrease of ADP–ATP phosphorus. Their fractionation procedure is not strictly comparable with ours, however, and the apparent discrepancy cannot be resolved without further investigation.

TABLE V

Developmental stage and phosphorus balance of R. pipiens ♀ × R. sylvatica ♂ gastrulae after 24 hours anaerobiosis, 14–15° C. For meanings of column headings, see section on Methods. Numerals following ± designate standard deviations. These values should be compared with those in part A of Table IV, obtained from R. pipiens embryos prepared from the same five clutches of eggs

Treatment	Shumway stage*	P_T	P_{AO}	P_I	No. expts.
Control	10	13.2 ± 1.0	0.57 ± 0.04	0.12 ± 0.01	5
Control	12	13.7 ± 0.7	0.55 ± 0.05	0.12 ± 0.01	5
Nitrogen	11	13.6 ± 1.0	0.56 ± 0.05	0.14 ± 0.02	5

* The stages assigned are those of simultaneously developing *R. pipiens* control embryos (Table IV, A).

Embryos in the hybrid *R. pipiens* ♀ × *R. sylvatica* ♂ fail to gastrulate, but remain alive during the whole time required for control *R. pipiens* embryos to reach the hatching stage (Moore, 1946; see review by Gregg, 1957). They are characterized by low respiratory rates and by low rates of aerobic and anaerobic glycolysis. The expectation that they might therefore find it more difficult than normal embryos to maintain esterified phosphorus stores under the stress of anaerobiosis was confirmed by the data of Barth and Jaeger. Our own experiments do not bear out this expectation, for they show (Table V) that hybrid embryos under anaerobiosis do not alter their phosphorus balance to a greater extent than *R. pipiens* controls. But it is not clear that these results are in genuine disagreement with those of Barth and Jaeger, for the reason stated at the end of the preceding paragraph.

Summary

1. *Rana pipiens* gastrulae treated with non-damaging concentrations of sodium azide or 2, 4 dinitrophenol for 24 hours at 14–15° C. exhibit a reversible retardation of morphogenetic movements, a diminished store of acid-soluble organic phosphorus, an elevated content of inorganic phosphorus and an unaltered total phosphorus content.

2. Anaerobiosis for 24 hours at 14–15° C. does not alter the phosphorus balance of *R. pipiens* gastrulae, or of gastrula-arrested hybrids of *R. pipiens* ♀♀ with *R. pipiens* ♂♂, beyond a slight elevation of the inorganic phosphorus level.

3. These results are discussed briefly in respect to the energy-requirements of the morphogenetic movements of gastrulation.

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RESPIRATION OF HOMOGENIZED EMBRYOS: RANA PIPIENS AND RANA PIPIENS ♀ × RANA SYLVATICA ♂[1]

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Embryos belonging to the hybrid *R. pipiens* ♀ × *R. sylvatica* ♂ cleave and blastulate normally, but the normal sequence of gastrulation movements does not occur (Moore, 1946). Such embryos remain alive, but suspended in a morphological state superficially similar to that of a very young gastrula. Before the occurrence of the developmental block, the respiration of hybrid embryos is quantitatively similar to that of normal *R. pipiens* controls, increasing exponentially with age; but for most of the period following its occurrence, their respiration is characterized by a function whose value is a constant (Barth, 1946). For an account of the attempts that have been made to analyze the biochemical and morphogenetic peculiarities of this hybrid, the reader is referred to the review by Gregg (1957).

The problem of explaining the respiratory peculiarities of gastrula-blocked hybrids is of course closely connected with that of constructing a theory to account for the exponential respiratory increase characterizing the pre-hatching development of normal embryos. In connection with this latter problem, suggested explanations have tended to fall into at least two classes: (1) those which postulate an increasingly rapid developmental synthesis of respiratory enzymes or substrates, and (2) those which assume a progressive increase in the structural availability of respiratory enzymes to their substrates. Each of these is supported by at least some of the available evidence. On the assumption that one or both of those types of explanation is well-founded, it is plausible to suggest two corresponding sorts of explanation to account for the post-blastula deficiencies of hybrid respiration: (i) that there is a failure to continue the synthesis of respiratory enzymes or substrates at a sufficient rate, or (ii) there is a failure of some developmental process which normally continues to increase effective contact of respiratory enzymes and their substrates.

In this paper we report the results of some simple homogenization experiments which it is hoped will have some bearing on these various questions.

Methods

Fertilizing, rearing and staging embryos. For fertilizing and rearing embryos, the following routine was adopted. Two separate sperm suspensions, one of *R. pipiens* sperm and one of *R. sylvatica* sperm, were prepared simultaneously in two fingerbowls, N and H. Half of the ripe eggs from a gravid *R. pipiens* were stripped into N and half into H. After two or three hours, the *R. pipiens* embryos in N were separated with scissors into small groups and distributed among several fingerbowls $N_1, \ldots N_n$; the hybrid embryos in H were similarly dispersed among several bowls $H_1, \ldots H_n$. Embryos in pairs of bowls (N_1, H_1) were reared at

[1] This investigation was supported in part by a research grant, No. A-1082, from the Public Health Service.

similar temperatures, ranging from 8° C. to 25° C., as convenient. Thus, the normal embryos in bowl N_1 served as controls for the hybrids in the corresponding bowl H_1. The medium in the bowls, 10% amphibian Ringer's solution without phosphate or bicarbonate, was changed daily. At the desired stages, obtained by reference to the tables of Shumway (1940), embryos were freed of their jelly coats with jeweler's forceps, re-staged, and homogenized. Hybrid embryos were assigned the stage-numbers characterizing the developmental stages of normal control embryos.

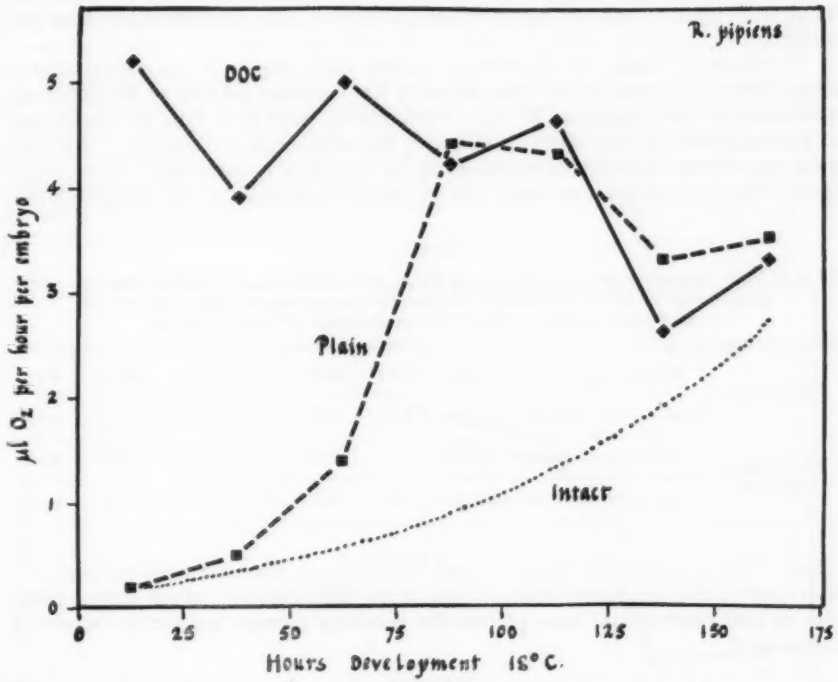

FIGURE 1. Respiration of homogenized *R. pipiens* embryos. Lower curve for intact *R. pipiens* embryos constructed from data of Moog (1944).

Preparation of homogenates. Cell-free breis were prepared by homogenizing batches of *n* jelly-free embryos in 0.05 *n*-ml. aliquots of suitable ice-cold media, using a high-speed homogenizer manufactured by the Lourdes Instrument Corporation. Two sorts of media were used routinely:

(a) 0.01 *M* phosphate buffer made up in 10% amphibian Ringer's solution without phosphate or bicarbonate, pH 6.8–7.0 Breis prepared in this medium will be called *plain-breis.*

(b) Medium (a) with the addition of 0.2% sodium deoxycholate (DOC). Breis prepared in this medium will be called *DOC-breis.* (We are indebted to Dr. W. H. Berg for suggesting the use of deoxycholate.)

Measurement of brei respiration. Within 10 minutes after preparation, 1.0-ml. aliquots of cold homogenate (about 20 embryos) were pipetted into 7-ml. Warburg flasks rigged for the measurement of oxygen uptake. After a period of temperature equilibration in the respirometer bath (about 10 minutes), manometer readings were begun and continued at 6-minute intervals for 45–60 minutes. The temperature of the respirometer bath was controlled at 24° C. The flasks were shaken continuously at a rate of 100–110 complete cycles per minute, at an amplitude of 6–8 centimeters. The rates of oxygen uptake were calculated from the readings taken during the first 30 minutes and are expressed as microliters (μl.) of oxygen per hour per embryo.

Treatment of data. In Shumway's tables, each stage s is correlated with a unique time $t(s)$, namely, the time required for a normal embryo to develop from fertilization to that stage, at 18° C. Furthermore, each $t(s)$ falls in exactly one of the successive 25-hour intervals following the moment of fertilization. We have made use of these correlations in presenting the results of measurements of the respiration of breis made from embryos with different environmental (temperature) his-

TABLE I

Effect of buffer concentration on respiration of plain- and DOC-breis, R. pipiens, stage 10. After equilibration period in respirometer, deoxycholate in appropriate buffer, pH 6.8–7.0, added from side-arm. Final brei concentration, 20 embryos per ml.

Plain-breis	Buffer concentration, molar	0.025	0.05	0.075	0.1	0.15	0.2
	μl. O_2 per hour per embryo	0.35	0.23	0.43	0.43	0.27	0.19
DOC-breis, 0.2% DOC	Buffer concentration, molar	0	0.01	0.02	0.04	0.07	0.08
	μl. O_2 per hour per embryo	7.8	8.0	7.9	6.4	6.6	6.9

tories. Thus, to construct Figure 1 and Figure 2, we have averaged for each 25-hour interval the respiratory rates of breis made from embryos whose stage s has a $t(s)$ in that interval and have plotted the resulting average against the interval's midpoint.

RESULTS

R. pipiens, plain-breis. Reference to Figure 1 will show that the average respiratory rate of plain-breis increases exponentially with the age of the embryos used in their preparation from an initial value of about 0.2 μl. O_2 per hour per embryo to a maximum value in the fourth 25-hour interval of about 4.5 μl. O_2 per hour per embryo, and thereafter tends to decline.

This result is in sharp contrast to that of Spiegelman and Steinbach (1945), who reported that the endogenous respiration of plain-breis prepared from embryos shortly after fertilization is already at a maximum. We are at a loss to explain the discrepancy, for we have been able to elevate the respiration of breis only by treatment with a detergent (see next paragraph), but not by altering the buffer concentration (Table I), nor, in preliminary experiments, by adding glycogen, glucose, hexose diphosphate, adenylic acid, magnesium, or various combinations thereof.

TABLE II

Effect of sodium deoxycholate concentration on respiration of homogenized R. pipiens embryos, stage 10–11. Breis and deoxycholate made up separately in 0.07 M phosphate buffer, pH 6.8–7.0. After equilibration period in respirometer bath, deoxycholate added from side-arm. Final brei concentration, 20 embryos per ml.

Final concentration of sodium deoxycholate, per cent	0	0.1	0.2	0.4	0.8
μl. O_2 per hour per embryo	0.12	2.8	6.6	5.6	4.4

R. pipiens, DOC-breis. The maximum respiratory rate attained by plain-breis, *i.e.*, that exhibited by embryos homogenized during the fourth 25-hour period of development, can be matched by that of deoxycholate-treated breis prepared from embryos of any lesser age (Fig. 1). Plain-breis and DOC-breis prepared from embryos older than this, however, respire at the same rate. The degree of respiratory elevation obtained earlier is a function of the concentration of DOC, 0.2% being the optimal concentration (Table II).

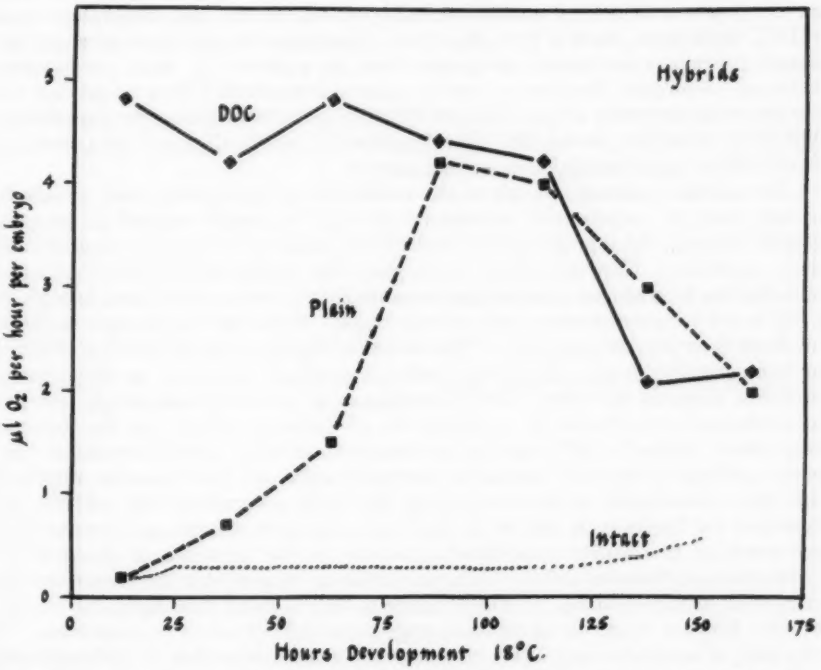

FIGURE 2. Respiration of homogenized gastrula-blocked hybrid embryos (*R. pipiens* ♀ × *R. sylvatica* ♂). Lower curve for intact embryos constructed from data of Barth (1946) and Moog (1944).

The curve for DOC-breis shown in Figure 1 is very similar to that reported for plain-breis by Spiegelman and Steinbach, although the general respiratory level is much higher and begins to decline somewhat later. We do not know if the stimulatory effect of DOC can be obtained with other detergents. However, we have found that plain-breis of early embryos respire at unusually high rates if they are prepared in Waring Blendor vessels freshly washed in Alconox. The effect wears off after several preparations without intervening cleansings with Alconox. This suggests that detergent is trapped in the bearings and leaks out slowly during the preparation of breis, but a systematic study of the question has not been made.

Hybrids, plain-breis and DOC-breis. The results of our measurements of the respiratory rates of plain- and DOC-breis made from hybrid embryos (Fig. 2) can be summarized very briefly: the respiration of such breis is quantitatively similar to that of corresponding breis made from normal control embryos, except perhaps in the seventh 25-hour period when the hybrids are moribund.

Discussion

There is no need to postulate a developmental synthesis of respiratory enzymes or substrates to account for the exponential rise of respiratory rate characterizing the development of normal amphibian embryos; for, as the high respiratory rates of DOC-breis show, there is from the outset of development enough respiratory machinery to support oxidation-rates greater than any exhibited by intact pre-hatching embryos. Similarly, there is no need to assume a synthetic failure to account for the abnormal constancy of post-blastula hybrid respiration, because the experiments with DOC-breis have shown that hybrid embryos at nearly all stages are potentially capable of as much respiration as normal controls.

For normal embryos, we adopt the conclusion of Spiegelman and Steinbach, namely, that the exponential respiratory increase is causally related to morphogenetic changes which progressively facilitate the union of respiratory enzymes and their substrates. Correspondingly, to explain the respiratory constancy of aging post-blastula hybrids, we assume that those changes somehow have been brought to a halt at the commencement of the gastrula stage. What sorts of changes might be involved is at present unknown. The elevation of embryonic respiration obtained by homogenizing in plain phosphate buffer, increasingly extensive as development proceeds, suggests that some cellular structures are becoming increasingly sensitive to mechanical disturbance or to alterations of chemical milieu, and the maximal respiration obtained at all stages by the treatment of breis with deoxycholate suggests a cellular component sensitive to detergent action: in both cases the mitochondria come immediately to mind because of their close association with oxidative enzymes of the Krebs cycle and of the hydrogen transport system, and because they are sensitive to isolative procedures, especially in the presence of deoxycholate (Siekevitz and Watson, 1956). It is reasonable to suppose that the extensive fragmentation of mitochondria by deoxycholate is only a more thoroughgoing version of what happens at any developmental stage to the mitochondria in plain-breis. In any case, it would be very interesting to study the mitochondria of differentiating embryos, and to compare those of hybrids with those of normal embryos.

The decline in the respiration obtainable from breis of late embryos may be explained by assuming, as Spiegelman and Steinbach do, a depletion of endogenous

respiratory substrates. In this connection, it is known that only half of the stored carbohydrate available at the time of fertilization remains in normal embryos at the time of hatching, although more than this remains in hybrids of the same age (Gregg, 1948).

Summary

1. The respiration of phosphate-buffered cell-free homogenates made from *R. pipiens* embryos increases exponentially with the age of the embryos up until the time at which they are in the tailbud stage, after which the rate declines.

2. Addition of 0.2% sodium deoxycholate elevates the respiration of homogenized embryos at any pre-tailbud stage to that of tailbud-breis, but has no effect upon that of breis of post-tailbud embryos.

3. The respiration of plain- or deoxycholate-treated breis is at all stages greater than or equal to that of intact embryos.

4. The respiration of breis (plain- and deoxycholate-treated) made from gastrula-arrested *R. pipiens* ♀ × *R. sylvatica* ♂ hybrid embryos is at all non-moribund stages quantitatively the same as that of control breis of normal embryos.

5. The implications of these findings are briefly discussed.

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A RATIONAL APPROACH TO THE PROBLEM OF CANCER CHEMOTHERAPY [1]

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In spite of the fact that over a long period of time, many workers in various parts of the world have been studying the curative effect of various chemical agents on cancer, there has been no very great effort to determine why these agents act as they do, and there is at present but little pertinent information as to the type of action these agents have on the cancer cell.

The problem is complicated by the fact that many of the very agents that have a definite curative effect can also act as carcinogenic agents. This was first noted by Haddow in 1935, and he and his collaborators have written a number of papers on the subject. In his very useful book on the "Biochemistry of Cancer," Greenstein (1954) frequently refers to this phenomenon as the "Haddow paradox." But neither Haddow nor Greenstein has any interpretation of the paradox. In 1951, Haddow in writing about carcinogens and substances which have a therapeutic effect on cancer writes (p. 264): "But in no case—a striking fact—do we know the place in the cell at which they act—whether the cell surface, the cytoplasm, the nuclear membrane, the nucleus itself—or the nature of the receptors with which they combine." And all that Greenstein has to suggest when he considers the problem is that (p. 278) "The capacity for intellectual flexibility combined with scientific care is one of the demands in the field."

In the past, most of the work that has been done on the chemical treatment of cancer and the theory back of such treatment has been done by chemists. And whatever efforts they have made toward interpretation have for the most part been inspired by chemical concepts built around the idea of some disturbance of metabolism. But some of the most powerful chemotherapeutic agents do not affect the growth of the cell. Thus in the presence of nitrogen mustard or its oxygen derivative, Nitromin, cells increase in size but do not divide (Bodenstein, 1947; Friedenwald, Buschke and Scholz, 1948; Sato, Belkin and Essner, 1956).

When a tumor arises in an organ or tissue the appearance of the neoplasm is always accompanied by a great increase in the number of cells that are dividing. Thus for example in the brain, there are normally no mitoses, but in a brain tumor there are great numbers of dividing cells. It is of course possible to believe that some increase in metabolism, or some change in metabolism, is the primary cause that started the tumor to develop. But it is just as possible to assume that the primary factor is an initiation of mitosis and that metabolic changes are a result rather than a cause.

[1] Supported by grants from the National Institutes of Health and from the American Cancer Society.

At the present time, we have a considerable body of information concerning the initiation of cell division, and there is also a satisfactory theory as to why and how cells can be made to divide. Moreover we have information as to the suppression of cell division and the reasons for it. Both the initiation and the suppression of cell division can be understood in terms of the behavior of the protoplasmic colloid, and the changes that occur in it that lead to the formation of the mitotic spindle. For a survey of this knowledge, see Heilbrunn's "Dynamics of Living Protoplasm" (1956). One of the strange facts about protoplasm is that the same agents which suppress its activity can also under certain conditions arouse it to activity. We thus have a parallel to the Haddow paradox, and indeed the initiation of cell division can be regarded as one example of the response to stimulation.

Can we hope therefore to interpret the chemotherapy of cancer and the Haddow paradox on the basis of a colloidal theory? Surely such an approach is worth looking into, especially in view of the fact that other types of interpretation have not as yet been successful.

In a long series of papers, most of them referred to in the book already cited, Heilbrunn and his co-workers have investigated the problem of the initiation and suppression of cell division from the standpoint of the colloidal properties of protoplasm and the changes that the protoplasmic colloid must undergo in order to form a mitotic spindle. The basic colloidal reaction of protoplasm is a clotting reaction similar in many respects to the clotting of blood. The protoplasm is in a state of equilibrium between the various factors which favor or induce clotting and those which prevent it. Before a mitotic spindle can form, a gelation must occur in the protoplasm. This is the mitotic gelation, and it can be induced either by a release of calcium from a bound state in the outer cortex of the cell or by the entrance into the cell of thrombin-like substances. Many agents prevent the mitotic gelation—among them, substances of a heparin-like nature.

Starting out from this point of view, we thought to investigate the action of certain chemotherapeutic agents which have been used in the experimental study of cancer as well as in clinical practice. The first question to be answered is whether or not these agents prevent the mitotic gelation. Then later we will consider the question of the Haddow paradox.

Materials and Methods

The cell we used as a test object was the egg of the worm *Chaetopterus pergamentaceus*. The eggs of this worm can readily be obtained at Woods Hole during the summer months. When the eggs are shed into sea water they are in the germinal vesicle stage. In 7 minutes (at 21° C.) the germinal vesicle breaks down and the first maturation division proceeds as far as the metaphase. Then all mitotic activity ceases until the egg is fertilized. Following fertilization the first maturation division is completed and the second maturation division immediately follows the first. As a result two polar bodies are given off and the egg then prepares for its first cleavage division. The mitotic spindle for this division appears at forty minutes after insemination (at 21° C.) and 50% of the eggs cleave at 56 minutes after insemination. Before the appearance of the mitotic spindle, the viscosity of the protoplasm increases markedly; this constitutes the mitotic gelation. Simultaneously with the appearance of the spindle the protoplasm becomes more fluid again so that

at the metaphase the viscosity is again low. Details of these changes are given by Heilbrunn and Wilson (1948). One advantage in using the *Chaetopterus* egg is that in any given lot the course of events in different individual eggs varies but little, and there is almost perfect synchrony.

Viscosity measurements were made with a hand centrifuge. At the present time it is not possible to buy suitable hand centrifuges. The ones we use are made for us by Mr. J. A. Appenzeller, technician of the Zoology Department of the University of Pennsylvania. They are adapted from a hand centrifuge sold by Sears, Roebuck and Co. and intended for the separation of cream from milk. In order to use these cream separators for our purposes it is necessary to fit them with a head which will hold glass tubes. The tubes we use have an outside diameter of 4 or 5 millimeters. When our centrifuges are turned at the rate of one turn of the handle per second, they give a force approximately 9,000 times gravity. We prefer to turn the handle once every two seconds; this gives a force one-fourth as great, that is to say, 2,250 times gravity. With a force of this magnitude, for most of the time between insemination and cleavage, it requires 7 seconds to move the granules in the *Chaetopterus* egg sufficiently so as to give the impression of zones. (The heavier granules move centrifugally and the lighter granules centripetally.) The number of seconds required to produce zoning is taken as an arbitrary viscosity value. At about 27 minutes after insemination (at 21° C.) the viscosity of the protoplasm begins to increase, and by about 30 or 32 minutes the viscosity has increased until it is approximately twice what it was before this mitotic gelation began. The viscosity then stays high until the spindle appears, a matter of about 8 minutes. It is during this time that tests of viscosity must be made if we are to discover if a given substance keeps the protoplasm fluid and prevents the mitotic gelation. These tests must therefore be made rapidly so that it is not possible to obtain definite values. But we can be sure that in the controls mitotic gelation has occurred if the viscosity is high enough so that it requires more than 8 seconds of centrifugal turning to cause an appearance of zones. Actually the viscosity during normal mitotic gelation is 14 in our arbitrary units. In the tables we record the viscosity of the control eggs as more than 8; it almost certainly is 14.

The nitrogen mustard used in our experiments was obtained from Sharp and Dohme in the form of a commercial preparation called Mustargen. This preparation comes in separate sealed vials, each of which contains 10 mg. of nitrogen mustard, that is to say methyl-bis(beta-chloro-ethyl) amine hydrochloride plus 100 mg. of NaCl. The contents of each vial were hastily dissolved in sea water, but because of the presence of the NaCl it was not possible to prepare solutions of high concentration, for such solutions would have been hypertonic and might have masked the effect of the nitrogen mustard.

Through the kindness of Dr. Edward S. Essner we were able to obtain Nitromin, an oxide derivative of nitrogen mustard manufactured by the Yoshitomi Pharmaceutical Industries of Osaka in Japan and distributed by Takeda Pharmaceutical Industries, also of Osaka. Nitromin is methyl-bis(beta-chloroethyl)amine-N-oxide hydrochloride. This compound is less toxic than nitrogen mustard and has been claimed to have better therapeutic value.

In using 6-mercaptopurine, we had difficulty. This substance is scarcely soluble at all in sea water. In order to obtain a solution we dissolved it first in a small amount of normal NaOH. Then strongly acidified sea water was added until a pH

TABLE I

Effect of Mustargen on fertilized Chaetopterus eggs

%	pH	Viscosity at 32–38 min.	% Cleavage
0 (control)	7.5	>8	94
0.1		8 or less	18
0.05		8 or less	20
0.025		8 or less	16
0 (control)	7.5	>8	94
0.1		8 or less	27
0.05		8 or less	32
0.025		8 or less	19

a little higher than that of sea water was reached. At this pH, microscopic observations showed the solution to be full of suspended material, so that we could not be at all certain as to how much of the substance remained in solution. In all of our experiments we were dealing with a saturated solution of unknown concentration, and we have the impression that in sea water 6-mercaptopurine is barely soluble.

RESULTS

When nitrogen mustard is dissolved in sea water the resultant solution has a much lower pH than does sea water. In our first experiments we made no attempt to neutralize the acid in our solutions, and the results of these experiments were therefore discarded. Table I gives the results of two experiments in which the Mustargen solution was made more alkaline by the addition of NaOH solution. In both experiments, the solutions were brought to a pH of 7.5 and were thus still somewhat less alkaline than sea water. However, in preparing dilutions from the 0.1% solution of nitrogen mustard, the dilutions were of course made with sea water so that in the lower concentrations of the drug, the pH was not very different

TABLE II

Effect of Nitromin on fertilized Chaetopterus eggs

%	pH	Viscosity at 31–35 min.	% Cleavage
0 (control)	7.9	>8	100
0.4		8 or less	0.5
0.3		8 or less	4
0.2		8 or less	9
0 (control)	8.0	>8	97
0.5		8 or less	0
0 (control)	7.8	>8	97
0.4		8 or less	0
0.3		8 or less	0
0.2		8 or less	2

from that of sea water. Moreover, a pH of 7.5 has but little effect on the protoplasm of *Chaetopterus* eggs.

The experiments with nitrogen mustard indicate that this substance keeps protoplasm fluid and prevents the mitotic gelation. It thus acts in the same way as do various other antimitotic substances previously studied by us (Heilbrunn and Wilson, 1950a, 1950b, 1956; Heilbrunn, Wilson and Harding, 1951; Heilbrunn, Chaet, Dunn and Wilson, 1954). The mechanism of this action will be discussed later.

Our experiments with Nitromin gave more striking results than those with nitrogen mustard. Like Mustargen, Nitromin when dissolved in sea water causes a substantial reduction in the pH. As before, we added enough NaOH to bring back the solution to a pH like that of sea water. The results obtained with Nitromin are shown in Table II. They show conclusively that this derivative of nitrogen mustard keeps protoplasm fluid and completely prevents the mitotic gelation. Very few of the eggs exposed to rather dilute solutions of Nitromin ever cleave.

As pointed out in the section on Materials and Methods, 6-mercaptopurine is very sparingly soluble in sea water, and sometimes we wondered if any of it went

TABLE III

Effect of 6-mercaptopurine on Chaetopterus eggs

%	pH	Viscosity at 30–35 min.	% Cleavage
0 (control)		>8	97
0.1	8.25	8 or less	49
0 (control)		>8	98
0.3	8.1	8	99
0.3 (exposure 30 min. before fertilization)	8.1	8	70

into solution at all. Our results with this substances are not very impressive; they are shown in Table III. The results we did obtain indicate clearly enough that 6-mercaptopurine tends to keep the protoplasm of the *Chaetopterus* egg fluid. This effect is more pronounced when the solution is a little more alkaline, presumably because at the higher alkalinity more of the substance stays in solution. Also at the higher pH the inhibition of cleavage was greater.

Nitrogen mustard, Nitromin and 6-mercaptopurine thus all have the same sort of effect on the protoplasmic colloid. All of them tend to keep the protoplasm fluid and prevent the mitotic gelation. In the past it has been shown many times that the same agents which prevent gelation may, in other concentrations, have quite the opposite effect (for references and discussion, see Heilbrunn, 1956). Fat solvent anesthetics, which keep protoplasm fluid and thus prevent response to stimulation, may in certain concentrations act as stimulating agents and when they do they induce a clotting or gelation of the protoplasm. Now it is proper to consider the prevention of cell division by agents which do not kill the cell as a form of anesthesia or narcosis, and indeed various anesthetic agents do prevent cell division. And the initiation of cell division can be regarded as a response to stimulation. All this being true, might it not be possible to show that with other concentrations of nitrogen

TABLE IV

Effect of ethyl urethane on fertilized Chaetopterus eggs

% Urethane	Viscosity 30–33 min. after fertilization	% Cleavage
0 (control)	>8	100
2	8 or less	0
1.5	8 or less	0
1	8 or less	0

mustard or Nitromin a gelation of the protoplasm could be induced and perhaps also an initiation of cell division? If we could show this, we would have a way of interpreting the Haddow paradox.

We did not attempt to do this experiment with nitrogen mustard, for the preparation of this drug that was available to us—Mustargen—contains ten times as much NaCl as it does nitrogen mustard, and if we made relatively concentrated solutions we would arrive at concentrations of salt which would in themselves cause the initiation of cell division. However, with Nitromin this difficulty does not exist. Accordingly, we tried the effect of a 1% and a 0.5% solution of Nitromin on unfertilized *Chaetopterus* eggs. Both of these solutions caused a marked increase in the viscosity of the protoplasm. In the weaker solution this increase (after 80 minutes) was at least two-fold; in the stronger solution the viscosity increase was even greater and the protoplasm seemed quite solid. In both cases the drug caused a vacuolization of the protoplasm. This is the type of reaction which Loeb (1913) called cytolysis, and it is a reaction commonly produced by agents which initiate division in marine eggs when these agents are used in too strong a concentration or for too long an exposure. However, in the one experiment we tried, we were not able to obtain any initiation of cell division. In this experiment the eggs were exposed to 1% and to 0.5% Nitromin for periods of 1, 2, 5, 10, 20, 30 and 60 minutes. Our failure to obtain initiation of cell division with the Nitromin solutions was not surprising, for although in every case when egg cells are stimulated to divide, the viscosity of the protoplasm in the interior of the cell is markedly increased, the reverse is not true; for an agent which tends to gel or clot the protoplasm may be too toxic to permit cell division to proceed. Thus in the work on Nitromin, there is only a partial explanation of the Haddow paradox. For though it is true that Nitromin can produce opposite effects on the protoplasmic colloid, we know only that it can suppress cell division and not that it can initiate it.

TABLE V

Effect of ethyl urethane on unfertilized Chaetopterus eggs

Time of exposure to 3% urethane	Viscosity
20 min.	11
32	13
54	13
Control (untreated)	8

TABLE VI

Effect of 3% ethyl urethane in initiating cell division of unfertilized Chaetopterus eggs

Exposure time in minutes	Exp. A: % cleavage after 3½ hours	Exp. B: % cleavage after 6 hours	Exp. C: % cleavage after 8 hours
5	0	0	3
10	0	16	24
15	0	16	50
20	15	40	55
25	26	80	50
30	37	88	45
35	26		29
40	20		34
45	11		20
50	13		23
55	10		22
60	4		6
Control	0	0.05	9

We thought therefore to try the effect of urethane; for this substance, which is known to act both as a carcinogen and as a chemotherapeutic agent for tumors, is presumably less toxic than Nitromin. In relatively weak concentrations, ethyl urethane suppresses cell division in the *Chaetopterus* egg. This is shown in Table IV. This table also shows that in concentrations which suppress cell division, the urethane keeps the protoplasm fluid and prevents the mitotic gelation.

Higher concentrations of urethane have quite the opposite effect. Thus when unfertilized eggs are placed in a 3% solution of urethane, the protoplasmic viscosity increases sharply, as is shown in Table V.

Moreover exposure to 3% urethane can, in a high percentage of cases, cause the egg cells to divide. We have done some experiments of our own to show this, but experiments done by Mr. Herbert Schuel are more complete than ours and we prefer to present them. They are shown in Table VI.

DISCUSSION

Clearly, then, one and the same agent in different concentrations can cause either initiation of cell division or suppression of cell division and these opposite effects are readily correlated with the action the reagent has on the colloidal state of the protoplasm in the interior of the cell.

The facts as we have reported them are so clear cut that they scarcely require additional comment. Therapeutic agents commonly used in the treatment of cancer can prevent cell division by keeping the protoplasm fluid. Some of these agents, when used in different concentrations, can have opposite effects both on the physical state of the protoplasmic colloid and also, in the case of urethane, on the end result. Here, then, we have a way of interpreting the Haddow paradox, and we are able to supply the information Haddow was so concerned about, namely on which part of the cell these agents act and what they do.

But the question immediately arises as to the mechanism of the paradoxical ac-

tion. In the case of urethane, the answer is rather obvious in the light of what we know concerning the way various anesthetic or narcotic agents act on protoplasm. This subject is discussed at some length by Heilbrunn (1956). Suffice to say here that these agents liquefy the cortical protoplasm and release calcium from it; the calcium thus released enters the interior of the cell and there causes a clotting reaction, which may lead either to excitation or to a complete vacuolization of the protoplasm and death of the cell. But fat solvent anesthetics not only tend to free calcium from the cortex, they also tend to prevent calcium in the cell interior from causing a clotting reaction. There is a large and growing body of evidence in support of these statements (see Heilbrunn, 1956). In addition it should perhaps be noted that ether, which in low concentrations keeps the protoplasm of sea urchin eggs fluid (Heilbrunn, 1920, 1925), can in higher concentrations induce cell division (Mathews, 1900; McClendon, 1910); in these higher concentrations it causes a clotting reaction in the protoplasm.

It is to be hoped that other investigators will join with us in approaching the problem of cancer chemotherapy from the standpoint of the colloid chemistry of protoplasm. At the present time there are so many excellent and well trained workers interested in the metabolic approach, and scarcely anyone concerned with the reasons why the protoplasmic colloid changes in such a way as to form a mitotic spindle. And yet, without mitosis there can be no cancer, and if there are relatively non-toxic ways of preventing mitosis, certainly this is a field that should be investigated on a large scale. Insofar as we know at present, neither the initiation nor the suppression of mitosis depends on any particular metabolic pathway and it certainly does depend on colloidal changes in the protoplasm. Antimitotic substances such as can be extracted from ovaries can indeed be used to cure mice inoculated with a lethal ascites tumor (Heilbrunn, Wilson, Tosteson, Davidson and Rutman, 1957) and in their therapeutic action on this tumor they are at least as effective as nitrogen mustard or Nitromin. Indeed more recent experiments have shown them to be decidedly more effective.

Summary

1. Nitrogen mustard, Nitromin, 6-mercaptopurine, and urethane suppress cell division in *Chaetopterus* eggs.

2. This inhibition of mitosis is due to the fact that these agents keep the protoplasm fluid and prevent the mitotic gelation.

3. In relatively high concentration, both Nitromin and urethane cause a gelation of the protoplasm and in these concentrations, urethane can initiate cell division in a high percentage of the eggs.

4. An interpretation is given of this paradoxical action of reagents in causing either liquefaction or gelation, either suppression or initiation of cell division.

5. The results are believed to provide an explanation of the Haddow paradox.

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FURTHER STUDIES IN THE BEHAVIOR OF COMMENSAL POLYCHAETES

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To date a number of studies have been made on the response specificity of commensal polychaetes (Davenport, 1950, 1953a, 1953b; Davenport and Hickok, 1951; Bartel and Davenport, 1956). In these studies a number of techniques to discern the presence of chemical responses to host have been employed. The subject of specificity and behavior in animal partnerships has recently been reviewed (Davenport, 1955).

During the summer of 1956 further investigations of the behavior of a number of polychaete commensals were conducted at the Friday Harbor Laboratories of the University of Washington. The authors wish to express their appreciation to the Director and staff of the Laboratories for their continued interest and support in these researches. The studies are currently continuing in the Marine Biological Laboratory of Santa Barbara College and have been supported since 1955 by a contract from the Office of Naval Research.

The preliminary investigations cited above had indicated the necessity to compare the behavior of populations of single species of facultative and obligate commensals of diverse host-habit, and to determine whether these populations showed different response specificity. The following studies were directed to that end.

The Facultative Commensal Podarke pugettensis Johnson

Material

The hesionid polychaete *Podarke pugettensis* provides a most interesting subject for behavioral studies. The worm is a facultative commensal, and there appears to be no discernible morphological difference between free-living and commensal members of the species. In the free state the species occurs in great numbers under certain conditions; one may at times collect as many as 15–20 per square yard on the mudflats of Garrison Bay, San Juan Island, Washington. In the Southern California region it may be collected as it settles out of the plankton by suspending open-mouth jars under floats in San Pedro Harbor (D. J. Reish, personal communication), while numbers of adults may be taken by scraping the under surface of floats in the same locality and in Santa Barbara Harbor. It may also be collected by removing large pieces of the growth from pilings, where it occurs near the wood surface deep among the shells of the gastropod *Aletes* and the pelecypod *Chama*. It occurs among the byssus threads of *Mytilus* on pilings. Under these conditions the worms do not appear to be associated with any particular organism, but they certainly seem to thrive in environments of extremely rich organic content. Free-living individuals will be found sporadically in many sorts of environments, particularly where there is rich mud, in the inter-tidal and subtidal. During the summer of 1956 ripe

swarming adults were taken at the night light at Friday Harbor for the first time; whether these had been free-living or had come from hosts could not be determined. Swarming has never been observed by us in Southern California.

In California, these worms are commonly associated with the web-star *Patiria miniata* (Brandt), on one individual of which as many as 15–20 may occur. In the Pacific Northwest they are equally common on the mud-star *Luidia foliolata* Grube. There may be considerable variation in the size of worms on both hosts, indicating repetitive colonization by different age classes. In the Puget Sound-Vancouver Island region they may occur on the cushion-star *Pteraster tesselatus* Ives and together with *Nereis cyclurus* Harrington commensal with hermit crabs (Berkeley and Berkeley, 1948). Steinbeck and Ricketts (1941) list the species as commensal with the starfish *Oreaster occidentalis* Verrill in the Gulf of California. That it may occur occasionally with *Pisaster ochraceus* (Brandt) is indicated by a single specimen in the collection of Dr. Olga Hartman, taken by Dr. S. F. Light at Dillon Beach, California. In spite of examining numerous specimens of the common starfish, from Puget Sound to Southern California, we have never found commensal polychaetes of any sort associated with it; there would appear to be a likelihood that the above case was fortuitous.

Method of investigating responses

A.

A choice-apparatus has been designed for the investigation of the possible role of chemical attractants in the regulation of partnerships, such as that between *Podarke* and *Patiria,* in which we have been unable to demonstrate in the individual commensal partner any sharply defined, objectively recordable response to the host (Bartel and Davenport, 1956). The apparatus consists of an aquarium with a central chamber surrounded by and connected by passages with six radially arranged chambers. It may be constructed out of latex as described in the above citation. Our use of the apparatus was as described except that a cover of plywood was added to reduce the possible effects of light. The latter factor was eliminated from consideration in any series of tests by the random selection of test chambers from the possible six. In all the experiments using this apparatus described below, the presence or absence of an attractant factor in one of the chambers among the radial six (the "critical" chamber) is indicated when probabilities, using the null hypothesis that distribution into the six chambers is the result of chance, indicate that either a significant or insignificant number of worms have moved from the central chamber into the critical chamber. Tests averaged from 8 to 12 hours.

Between each test in a series in any experiment the apparatus was washed. Host animals were generally housed during tests directly in one of the radial chambers, but in certain tests indicated below, when host animals were very large, they were housed in a clean, redwood and glass aquarium and the water therefrom siphoned into a radial chamber of the choice-apparatus.

B.

Prior to employing the above described choice-apparatus, Bartel and Davenport (1956) had found, by the simple expedient of placing in dishes large numbers of free-living and commensal *Podarke* together with *Patiria,* that toward this host

there is a marked difference in behavior in the two populations; the commensals gathered on the star while none of the free-living worms did. It seemed wise to repeat this experiment in Puget Sound, using both the free-living worms and those commensal with the common host of that region, *Luidia foliolata*. As an experimental animal in behavior studies this starfish presents difficulties; it readily autotomizes its arms when handled or placed in a confined space and hence is not well suited to the latex choice-apparatus. At the same time it is so large that one cannot readily place it in a dish or tray with commensals. We therefore employed a large cement water table (internal dimensions 3′ × 5′ × 3″) in which the starfish could wander freely and "pick up" commensals or in which one could confine the star to a limited space so that the commensals had to "find" it (Fig. 1). In order to so confine the starfish we simply placed a plywood "T" in the table as shown, which would allow free movement of water or worms under its parts but which would

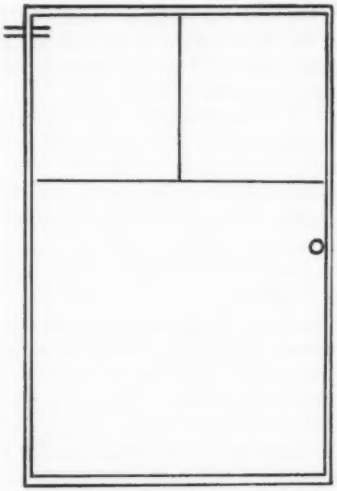

FIGURE 1. Plan of water table.

trap the star in one corner. Water was introduced at a very slow flow in one corner and drained out at the point shown. One introduced experimental worms at random at the lower end of the table.

This apparatus lent itself well to the study of the specificity of response in partnerships in which evidence for a chemical attraction effective at a considerable distance from the host had already been presented (*Arctonoë-Evasterias*, etc.—Davenport, 1950), and also made it possible to conduct tests concurrently with those using the latex apparatus, likewise testing the responses of a large sample of worms in a single test run.

Experiments

Experiment No. 1. Will commensal worms gather on the host *Luidia* when both have the freedom of the water table?

After a time duration of approximately eight hours, 23 out of 36 introduced worms (59%) had moved onto the starfish.

Experiment No. 2. Will commensals, introduced at random at the lower end of the table, find the host if it is trapped at the opposite end?

After a time duration of approximately nine hours, out of 23 worms introduced 13 had found the starfish (56%).

Experiment No. 3. Will free-living *Podarke* (Garrison Bay) gather on *Luidia* when both have the freedom of the water table?

After a time duration of approximately eight hours, out of 32 worms introduced *none* had moved onto the starfish.

Experiment No. 4. Can an attraction for commensal worms be demonstrated in the latex choice-apparatus if water is siphoned from a large aquarium containing the host *Luidia* into one of the six radial chambers?

Six *Luidia* were placed in a large redwood aquarium and three tests followed a control. In the control test with no starfish water in the system, 18 out of 23 worms made a choice and the distribution in the radial chambers was random. In the first test to starfish water, out of 25 worms introduced, 23 made a choice and of the 23, 14 entered the critical chamber ($P < .001$). In the second test all of 20 worms made a choice and of these 8 entered the critical chamber ($P < .01$). In the final test out of 25 worms 11 made a choice, of which 9 entered the critical chamber ($P < .001$).

It is clear that an attraction can be demonstrated with the *Podarke-Luidia* partnership in the latex apparatus. The above data compare very well with those obtained by Bartel and Davenport (1956) with the *Podarke-Patiria* partnership in California, in which two tests gave probabilities of $< .001$ (24 out of 68 and 23 out of 53 entering the critical chamber).

Experiment No. 5. Are free-living worms attracted to a radial chamber into which water from an aquarium containing *Luidia* is siphoned?

In a control test with no starfish water in the system, 16 out of 22 worms made a choice and the distribution was random. In four tests in which 15 out of 25, 16 out of 20, 17 out of 20 and 17 out of 24 made a choice when starfish water was in one of the chambers, the distribution was still purely random. In a single test against starfish water when 16 out of 24 worms made a choice, 8 entered the critical chamber ($P < .01$).

Since the above results were not consistent, further tests were indicated to determine whether or not the release of metabolites in test chambers may occasionally cause free-living worms to distribute themselves unequally in the choice-apparatus, in spite of the fact that under conditions more nearly approaching natural ones, they do not gather on *Luidia* (Experiment No. 3 above).

Experiment No. 6. Can closer propinquity to starfish (and therefore possibly greater concentration of metabolites) perhaps be the answer to the unequal distribution that may occur when free-living worms are tested in the choice-apparatus against *Luidia?* With considerable difficulty a single small *Luidia* was obtained for testing and one test completed with the starfish directly in one of the radial chambers before it autotomized its arms. In this test when 23 out of 30 worms made a choice, 8 entered the critical chamber ($P < .01$).

Experiment No. 7. A further series of tests were conducted at a later date to see whether free-living worms from a different environment than those used in Experiments 5 and 6 might distribute themselves in a non-random fashion when a host was in the system. Free-living *Podarke* from harbor floats in Santa Barbara were tested against the host starfish of California, *Patiria miniata*. In four out of five tests of this kind the distribution was random, but in one, when 19 out of 27 worms made a choice, 10 entered the critical chamber ($P < .001$).

Experiments 5, 6 and 7 have all given an indication that under certain conditions the behavior of free-living worms may be so affected by the presence of a host starfish in the system (perhaps by some metabolite) that their distribution will be non-random. However, they certainly do not respond positively as consistently as their commensal relatives.

Experiment No. 8. It has been demonstrated that commensal *Podarke* show a positive response to the host *Luidia* (Experiments 1, 2, and 4). Will worms from *Luidia* respond to the alternate host *Pteraster tesselatus?*

In two tests in which water from a redwood aquarium containing a single large *Pteraster* was siphoned into one of the six chambers, samples of 19 and 20 worms distributed themselves in a random fashion. But when a smaller *Pteraster* was placed in a radial chamber directly, in one test when 18 out of 23 worms made a choice, 9 entered the critical chamber ($P < .01$) and in the second test when 31 out of 36 worms made a choice, 16 entered the critical chamber ($P < .001$).

Here again propinquity may be a factor, and perhaps the great secretion of mucus produced by handling this starfish may have been a factor in preventing a response in the first two tests, when the starfish was at a greater distance.

Experiment No. 9. How specific is the response of commensals from *Luidia* in the choice-apparatus? Will the commensals respond to non-host starfish?

In a test against *Mediaster aequalis* Stimpson, when 24 out of 31 worms made a choice, 9 entered the chamber containing the host ($P < .01$). In a test against *Pisaster ochraceus* (Brandt) when 9 out of 19 worms made a choice, 5 entered the critical chamber ($P < 1.0$) and in a test against *Evasterias troschelii* (Stimpson) when 17 out of 27 worms made a choice, 11 entered the critical chamber ($P < .001$).

Apparently no response specificity can be demonstrated in the latex choice apparatus when one tests *Podarke* commensal with *Luidia* in Puget Sound.

Experiment No. 10. Do California *Podarke* commensal with *Patiria* show a similar non-specific response in the latex choice-apparatus?

In a series of six control tests against the host alternated with tests against non-host stars, distributions giving probabilities of $< .001$ were obtained in five, while in one test the distribution was random. In the series of 15 tests against *P. ochraceus* (Brandt), *P. giganteus* (Stimpson), *Pycnopodia helianthoides* (Brandt) and *Dermasterias imbricata* (Grube), when samples of from 10 to 44 commensals were used in a single test, all but one test gave completely random distributions. In one test against *P. giganteus* 19 out of 33 worms making a choice entered the critical chamber ($P < .001$).

There would appear to be a marked difference in the response specificity demonstrable in the choice apparatus between worms commensal with *Luidia* in Puget Sound and worms commensal with *Patiria* in California, the latter demonstrating a greater specificity.

The Obligate Commensal, Arctonoë fragilis (Baird)

Material and methods

The polynoid commensal *Arctonoë fragilis* has been studied previously by the authors (citations above). In the first experiments in which it was demonstrated that a marine commensal would respond positively to sea water which housed its host, a Y-tube olfactometer was used, but no detailed studies of response-specificity were made. Such apparatus does not lend itself readily to investigations of specificity of response, since large samples of commensals cannot be tested at once. As it had already been demonstrated that these worms showed an "overt" response to sea water from their host even at a distance, there appeared to be no advantage in employing the latex choice-apparatus. The use of similar water-table tests as described above (Fig. 1) was in order.

A. fragilis has been listed as commensal by Pettibone (1953) with the following asteroid hosts: *Evasterias troschelii; Leptasterias aequalis* and *L. hexactis; Orthasterias koehleri; Pisaster ochraceus; Solaster dawsoni* and *Stylasterias foreri.* We collected and used in the experiments below a large number of worms commensal with *Evasterias,* a few with *Orthasterias* and one (?) with *Solaster dawsoni.* It is unfortunate that cross-specificity studies are made difficult by the fact that it is almost impossible to collect a working sample of commensals from any other host than *Evasterias.* The Berkeleys tell us that at Nanaimo large numbers of *Orthasterias koehleri* can be collected in the inter-tidal zone in winter and early spring; in summer they can only rarely be so collected. Our few specimens of *Orthasterias* were taken in dredges and with the aqualung. It may in fact be possible in the future to compare the behavior of populations of *A. fragilis* from *Evasterias* and *Orthasterias,* by conducting winter experiments. The value of making a thorough comparison of the behavior of two or more separate populations of a single commensal species which inhabits several hosts is obvious. The brief preliminary tests presented below give evidence that the results of such experiments would be most interesting.

Experiment No. 11. Prior to running cross-specificity tests with the two populations of *Arctonoë* available, it was necessary to run a control experiment to determine whether under the conditions of the water table, *Arctonoë fragilis* (commensal with *Evasterias*) would show a response to non-host stars. In four control tests against *Evasterias,* run in alternation with tests against non-host stars, fifteen worms were used in three and fourteen in one. Tests had a duration of not less than nine hours. In the first three 12 out of 15 (79.9%), 10 out of 15 (66.6%), and 10 out of 15 "found" the "trapped" host. In the one test using 14 worms, 13 "found" the host (92.8%). In single tests using fifteen worms against *Pisaster ochraceus, Luidia foliolata, Mediaster aequalis, Hippasteria spinosa* and *Dermasterias imbricata* no worms "found" or moved onto the "trapped" non-host.

Commensal *Arctonoë,* therefore, demonstrate a rather precise response specificity in the water-table.

Experiment No. 12. It appears that commensals from *Evasterias* do not, as one might expect, demonstrate in the water-table a response to stars with which the species is not associated, but what sort of behavior would the worms show in relation to alternate hosts? Will the worms, regardless of host habit, respond to alternate hosts?

To answer this question we presented mixed samples of *Arctonoë,* some from *Evasterias* and some from *Orthasterias koehleri,* with opportunities to "find" each host in the water table. Will each host "sort out" the correct commensals? Unfortunately, because of the above-mentioned difficulty of finding *Orthasterias* we were able to collect only two specimens of *Arctonoë* from this host. In the following experiments these worms were lightly stained in indulin in order to distinguish them from the sample collected from *Evasterias.*

In a series of three water-table tests of this mixed population against *Evasterias* a total sample of 16 worms were used in each. In the first two tests 15 *Evasterias* commensals "ran against" one *Orthasterias* commensal. At the end of nine hours in both tests 10 (75%) of the *Evasterias* commensals had "found" their host while the single *Orthasterias* commensal was still wandering free. In the third test 14 *Evasterias* commensals were "run against" two *Orthasterias* commensals. At the end of nine hours all but one of the *Evasterias* commensals had "found" the host while the two *Orthasterias* commensals were still wandering free.

In two tests against *Orthasterias* a mixed sample of 15 worms was used in the first and of 17 worms in the second. In the first, out of 14 worms from *Evasterias* 13 were still wandering free after nine hours, while the single *Orthasterias* worm had "found" its host. In the second test at the end of the same time, out of 15 *Evasterias* worms none had moved onto the *Orthasterias,* while of the two *Orthasterias* worms, one had "found" the host.

These preliminary experiments against alternate hosts were conducted with a much smaller sample of worms than one would desire and it is hoped that at some time such tests can be repeated with balanced samples. But the tests give an indication of what may be a significant fact. There may, if such responses are not conditioned during development, be good physiological or behavioral races inhabiting different hosts within single commensal polychaete species. That this may be the case was further indicated by a brief experiment in which we tested a mixed population from *Evasterias* and *Solaster.* Accurate identification of the three species of *Solaster* with their two commensal species of *Arctonoë* (*S. stimpsoni* and *S. endeca* with *A. vittata* and *S. dawsoni* with *A. fragilis*), may be difficult. This is particularly true in the case of the worms, in which two species inhabiting closely related hosts may resemble each other greatly; identification can at times only be made by dissection which renders the animals useless for behavior experiments. However, we believe our identification of a single *A. fragilis* on *S. dawsoni* to be correct. When a mixed population consisting of 16 *A. fragilis,* one from *Solaster* and 15 from *Evasterias,* were tested in the water-table against *Solaster,* not one of the *Evasterias* worms moved to the star and yet the single *Solaster* commensal quickly "found" its host.

The Obligate Commensal Arctonoë vittata (Grube)

Material and methods

The polynoid *Arctonoë vittata,* closely related to *A. fragilis,* has perhaps the most interesting variation in host-habit of all the members of the genus. It colonizes certain asteroids, amphineurans, gastropods and polychaetes and within these groups shows a rather precise specificity (Pettibone, 1953). For this reason one might suppose that there could hardly be a commensal better suited to studies of response

specificity. Unfortunately, however, as with *Arctonoë fragilis,* it is very difficult to obtain large enough numbers of commensals from each host to make good studies of cross-specificity. In addition to the difficulty in collecting diverse populations of this commensal, one faces the problem of the general inactivity of the worm, which makes studies using a Y-tube or an open water-table tedious in the extreme. It was found, however, that a sample of animals distributed themselves well overnight in the latex choice-apparatus. The following questions were asked and to a certain extent answered, using a single population of worms from the key-hole limpet *Diadora* in the latex apparatus, according to the technique described above.

Experiment No. 13. Will commensals from *Diadora* show a response to the host in the choice-apparatus?

In eight tests samples of from 11 to 29 worms were tested against a group of six limpets in a radial chamber. In two of the eight tests the worms distributed themselves in a random fashion but in six of the tests enough worms chose the critical chamber to give probabilities of $< .001$, $< .01$, < 1.0, < 1.0, $< .001$, and $< .1$.

Experiment No. 14. Do worms commensal with *Diadora* show a response to alternate hosts? Sample alternate hosts tested were the starfish *Luidia foliolata* Grube, *Solaster stimpsoni* Verrill, and *Dermasterias imbricata* Grube; the chiton *Cryptochiton stelleri* Middendorf; and the gastropods *Acmaea mitra* Eschscholtz and *Fusitriton oregonense* (Redfield). Large hosts (*Luidia, Solaster, Dermasterias, Cryptochiton*) were housed in a clean redwood aquarium and the water siphoned from this into one of the radial chambers of the choice-apparatus. Small hosts (*Acmaea, Fusitriton,* small *Cryptochiton* and *Dermasterias*) were placed directly in a radial chamber.

In thirteen tests against these alternate hosts using samples in each of from 13 to 27 commensals the worms distributed themselves in the radial chambers in a random fashion. In a single test of the three against *Fusitriton,* when 20 worms made a choice, 8 entered the critical chamber ($P < .1$), while in the other two tests the distribution, although in both cases the greatest number of worms making a choice entered the critical chamber, gave probabilities $> .1$.

This series of tests indicates that the population of worms commensal with *Diadora* shows under these experimental conditions a rather precise response specificity. Unfortunately, the time duration of this experiment precluded our going further than analyzing the response to an array of available hosts. Certainly, a longer series of tests should be made against *Fusitriton* to determine whether toward this animal, which in some places occupies the identical environment from which the host *Diadora* may be collected, the worms show a constant response.

Discussion

Since the initiation of the study of the specificity of response of polychaete commensals in the summer of 1949, a number of different forms have been investigated. It has been our continued aim to try to correlate this *response* specificity with the known *host* specificity of the species or races. In our effort to make comparisons we have been continually faced with difficulties, some of which have been insurmountable. Among these is the fact that it is extremely difficult to collect large enough samples of worms for such studies in those most interesting species which show within themselves a diversity of host habit; in most such species the worms

will, in one locality, occur commonly on one host but very rarely on others. A difficulty encountered in making comparisons between the behavior of different species has been that, as one might expect, not all species exhibit the same sort of response, some showing as individuals a marked or overt response to factors from the host coming from a distance and others merely "accumulating" on or near the host after a passage of time. With such forms as the latter it has been necessary to design special techniques quite different from those used in studies on the former to discern whether or not there is in actuality any response to chemical factors coming from the host. The use of entirely different techniques has made a comparison of results difficult. Some differences in response specificity may turn out to be more apparent than real when some technique has been developed which lends itself equally well to the study of the responses of forms which appear to differ in behavior. Recently we have begun an analysis of the behavior of individual polychaete commensals when under the influence of host factor, using apparatus which may give us some truly comparable data even when studying animals of greatly differing activity or sensitivity.

However, it may be possible at this time, in spite of the above-mentioned difficulties, to make some brief general observations on response specificity in commensal polychaetes.

There would appear to be different categories of response specificity. There is a range of behavior, from the sort which is exhibited by species or populations within species that respond to their host alone, to the sort in which the commensals appear to have no chemical discernment and respond, at least under experimental conditions, to many non-host animals. Specificity of host habit is by no means an indication of specificity of response in experimental apparatus. As an example of the first category which exhibits precise response we have *Arctonoë fragilis* and its behavior in relation to *Evasterias, Orthasterias* and *Solaster*. But there are also species in which populations from one host may give a similar precise response to some, but not necessarily all, alternate hosts, regardless of the absence of any taxonomic affinity between the hosts to which they do respond (*Harmothoë lunulata* from the brittle-star *Acrocnida brachiata* vs. its host and the alternate eunicid *Lycidice ninetta* —Davenport, 1953b). Among such species of diverse host habit there may be a population occurring on one of the array of hosts which responds to its host alone, in spite of the fact that other populations of the same species respond to several alternates (*Harmothoë lunulata* from *Leptosynapta inhaerens*). A further category consists of those species which respond with the same intensity to the known alternate hosts but with reduced intensity to a number of non-host relatives of their hosts (*Acholoë astericola* from *Astropecten irregularis* vs. its host and the alternate *Luidia ciliaris*, as well as non-host stars—Davenport, 1953a). Finally we have a category which, though somewhat unexplainable, can be demonstrated to exist even when using a standard technique. In some facultative commensals there appear to be populations (*Podarke* on *Patiria*) which show a precise response specificity to their host alone and others (*Podarke* on *Luidia*) which seem unable to discern the difference between their host and other non-host animals.

It is therefore quite apparent that it is pressing to determine, particularly in forms such as *Podarke*, whether responses are inherited or conditioned. Although it would seem difficult to imagine a mechanism whereby such a host response could be conditioned in forms such as *Podarke*, the early stages of which (in the labora-

tory) remain in the plankton for some 30 days, nevertheless only successful breeding and settling experiments will give us the answer.

Summary

1. A new water-table test apparatus for the investigation of commensal response behavior is described.

2. Evidence is presented that the two populations of the facultative commensal *Podarke pugettensis* (Polychaeta: Hesionidae) which may be termed "commensal" and "free-living" differ markedly in their response to host animals, the commensal worms showing a strong tendency to respond positively to the host and the free-living worms not doing so.

3. Commensals with the starfish *Luidia* in Puget Sound appear to respond with almost equal intensity to other non-host animals (the response is not specific), while commensals of the star *Patiria* in Southern California show a more precise and specific response. This behavioral difference remains unexplained.

4. The behavior of three populations of the obligate commensal *Arctonoë fragilis* (Polychaeta: Polynoidae) was compared. Evidence is presented that each population (one commensal with the star *Evasterias*, one with the star *Orthasterias* and one with the star *Solaster*) shows a response to its host alone.

5. The response behavior of *Arctonoë vittata* (Polychaeta: Polynoidae), an obligate commensal of diverse habit, was investigated in relation to a number of its alternate hosts.

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ON THE MORPHOLOGY OF THE NEPHRIDIUM OF NEREIS VEXILLOSA GRUBE [1]

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It has been well established that certain of the Nereidae are capable of surviving in waters of low salinity. In the field this is demonstrated by their invasion of brackish and even fresh waters (Johnson, 1903; Hartman, 1938; Smith, 1950, 1953, 1956), and has also been suggested in the experimental work of some investigators (Schlieper, 1929; Nomura, 1930; Jürgens, 1935; Sayles, 1935; Beadle, 1937; Ellis, 1937; Topping and Fuller, 1942; Krishnan, 1952; Smith, 1955). In spite of the fact that many physiological studies have been carried out on various nereids, only a few morphological descriptions of the presumed osmoregulatory organ of these annelids, the nephridium, are to be found in the literature.

The first detailed description of nephridial morphology was made by Goodrich (1893) on *Nereis diversicolor*. He found three sections along the length of the nephridial tubule, each grading into the next. The sections varied in respect to the presence or absence of cilia, the diameter of the tubule lumen, and the extent of convolution of the tubule.

Fage (1906) studied *Perinereis cultrifera*, confining his work to living material. He also found areas of ciliation, but these differed from those observed by Goodrich in *N. diversicolor*. Much later, in his extensive review of observations of nephridia and genital ducts, Goodrich (1945) re-stated his earlier findings but added little to them. In his work on *Lycastis indica*, *Nereis chilkaensis*, and *Perinereis nuntia*, Krishnan (1952) made a study of the nephridia of each species and compared them with respect to vascularization and size, relative to body size.

Because of the paucity of adequate morphological studies on nereid nephridia, it was felt that further study was in order, to provide a better basis for physiological work, and for later studies of comparative functional morphology.

Materials and Methods

Specimens of *Nereis vexillosa* utilized in this study were obtained from a breakwater at the Berkeley Yacht Harbor, in San Francisco Bay, California. Although no salinity determinations were made at this time, the annual salinity range for this area is from 26.3 to 32.4‰ (approximately 73 to 90% sea water), according to Sumner *et al.* (1914) and Miller *et al.* (1928).

The worms were relaxed by gradual addition of 30% ethyl alcohol, fixed in Bouin's fixative and serially sectioned at eight micra. They were then stained with

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Harris' haematoxylin and counterstained with eosin Y. Other fixatives and stains were utilized, but these gave poor results.

In order to obtain a graphic representation of the canal as it passed through the nephridial mass, a plaster-of-Paris reconstruction was made. Camera lucida drawings were transferred to sheets of paraffin of proper thickness, and as the replica was built up, the lumen of the canal was hollowed out. Later, the canal was filled with plaster, and the surrounding paraffin was melted away.

Nephridial Morphology

The nephridia of *Nereis vexillosa* are paired organs in the coelomic cavity of each segment, just lateral to the ventral longitudinal muscle bundles, near the base of each parapodium. Within the broad base of attachment of the nephridium to the body wall, the nephridial canal opens to the exterior by way of the nephridiopore

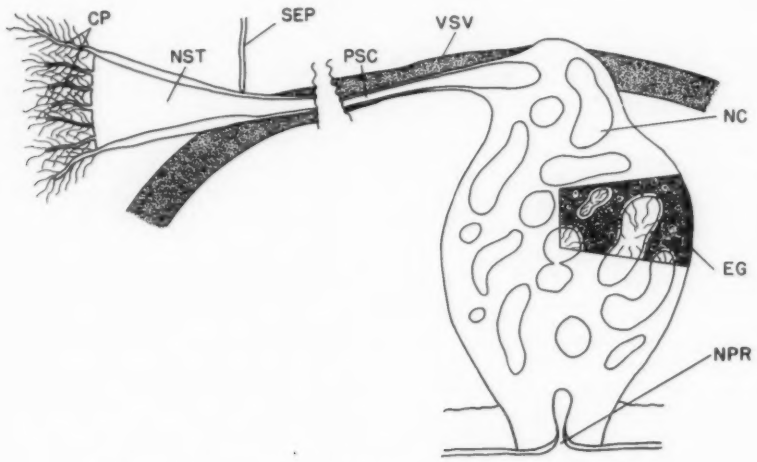


Figure 1. Schematic diagram of a nephridium of *Nereis vexillosa*.

(Figs. 1, 2, 6, NPR). The internal opening of the nephridial canal, the nephrostome (Figs. 1, 3, 5, NST), is found at the end of an anterior extension of the canal (the post-septal canal, Figs. 1, 3, PSC) which leaves the mass of the nephridium and passes anteriorly, through the inter-segmental septum (Figs. 1, 3, 5, SEP), to the next segment.

Externally, the nephridium is a discrete mass of tissue, varying from globose or pyriform to irregular in shape. The surface may be ridged to some extent, because of the passage of the nephridial canal close to the surface. In mature worms of from 55 to 70 segments (*ca.* 70 mm. long when relaxed) nephridia were approximately 250 micra at their widest and about the same dorso-ventrally. They measured nearly 200 micra through their antero-posterior axis, exclusive of the post-septal canal and nephrostome which, in themselves, were about 300 micra in length.

In section, the convoluted nephridial canal is seen as many perforations in the

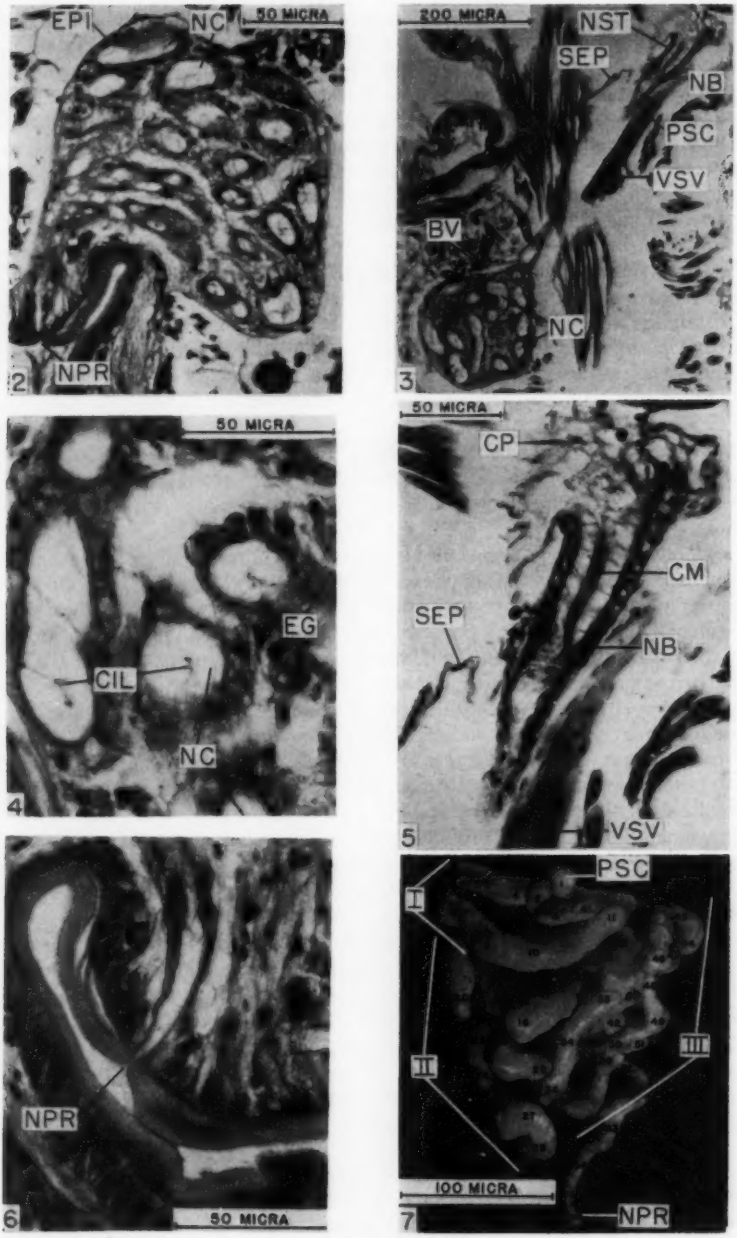
nephridial tissue (Figs. 1, 2, 3, 4, NC). A fairly discrete wall often lines the tubule, although, usually, the boundaries of these cells are difficult to resolve (Fig. 4). The surface of the nephridium is covered by a single, very thin layer of squamous coelomic epithelium cells (Fig. 2, EPI). It is well to point out that, as far as is known, all nereid nephridia have this general configuration, *i.e.*, a convoluted canal in the nephridial mass. An apparent contradiction to this fact occurs in a recent text (Prosser *et al.*, 1950, pp. 17–18) where it is stated that ". . . the nephridium of *N. cultrifera* is a simple sac." This is justified by a figure modified from Jürgens (1935) which had been redrawn from the work of Fage (1906). Fage's original figure was a surface view of the nephridium of *Nereis cultrifera*, and in subsequent copyings, the delicate shadings which showed surface texture were lost, and the figure evolved to that of an optical section of the nephridium. The fate of the figure notwithstanding, Fage (1906, p. 338) described the nephridium as a ". . . masse spongieuse, perforée en tous sens par un grand nombre de canaux"; therefore, it is certainly not a simple sac. The matrix of the nephridium is a highly vaculated, syncitial, network of loose connective tissue, which serves to bind the convolutions of the nephridial canal.

Nuclei are of two types, a smaller kind, rich in chromatic material ($3 \times 5\ \mu$), and a larger, clearer kind ($5 \times 10\ \mu$). Nuclei of both types are usually found in or near the canal walls, and only occasionally are they seen isolated in the matrix tissue (Figs. 2, 3, 4).

No blood vessels have been noted within the nephridial mass, and in only two places is the nephridial system approached by vascular elements. One blood vessel passes over the anterior face of the nephridium (Fig. 3, BV) and another, the ventral segmental vessel, lies along the post-septal canal (Figs. 3, 5, VSV). In neither case is the association intimate, and there is little opportunity for the transfer of materials from one structure to the other. Although neither Goodrich (1893) nor Fage (1906) mentions the relationships between the nephridium and the vascular system, Krishnan (1952) has indicated that in *Lycastis indica*, *Nereis chilkaensis*, and *Perinereis nuntia*, blood vessels are found in close association with nephridia. He also points out that the extent of nephridial vascularization is inversely related to the salinity of the environment.

Occasionally, eosinophilic granules have been seen in the tubule walls and the matrix tissue (Fig. 4, EG). There is no special distribution along the length of the canal, and no special accumulations in the nephridial mass. Goodrich (1893) mentioned minute granules in the cells of the tubule wall in *N. diversicolor* and considered these to be composed of excretory materials. Fage (1906) observed that with the addition of neutral red to the sea water bathing the freshly-dissected nephridia of *Perinereis cultrifera*, granules were formed which were similar to those which were observed in untreated nephridia, and which Fage terms, excretory granules (grains d'excrétion).

The post-septal canal (Fig. 3, PSC) is produced anteriorly as an extension of the nephridial canal, and is covered by the same thin squamous layer which invests the nephridium. Nuclei are uniformly scattered along its length and are concentrated in a band at the level of the septum (Fig. 5, NB). Anterior to the septum, the post-septal canal enlarges and gives rise to the funnel-shaped nephrostome. The lateral margin of the nephrostome is slightly recurved, and around the entire rim, there are numerous cytoplasmic processes (Fig. 5, CP).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FIGS. 2-7.

At the terminal end of the nephridial canal, the wall of the lumen thins and becomes continuous with the invaginated cuticle of the outer surface to form the nephridiopore (Fig. 6, NPR). In this respect, the structure of this area differs from that of *N. diversicolor* and *N. chilkaensis*, for Goodrich (1893) states that in *N. diversicolor* the wall of the tube pierces the epidermis, and Krishnan (1952) presents a figure showing the same condition in *N. chilkaensis*.

By means of camera lucida drawings, the entire course of the convolutions of the nephridial canal was followed from the nephrostome to the nephridiopore. It was then possible to ascertain the extent of ciliation within the lumen of the canal (Fig. 4, CIL). It was seen that the ciliation of the nephrostome is extremely heavy, and forms a tightly wound swirl in the throat of the nephrostome (Fig. 5, CM). The heavy ciliation is maintained throughout the rest of the post-septal canal, and gives a characteristic "star" or "wagon wheel" aspect to transverse sections of this structure. The ciliation of the portion of the canal included within the nephridial mass is constant, but not uniform. No obvious areas of heavy or sparse ciliation, such as were noted by Goodrich (1893) in *N. diversicolor*, have been observed here, and in general, the midportion is only slightly more heavily ciliated than either end. In the region of the nephridiopore (Fig. 6), the canal is devoid of cilia for about the last 40 micra of its length. It has also been noted that in *N. vexillosa* the cilia are never attached on only one side of the lumen as Goodrich reported in *N. diversicolor*. In addition, no tufts of cilia, such as Fage (1906) has described in the nephridial canal of *Perinereis cultrifera*, have been seen here.

The plaster reconstruction (Fig. 7) shows that after the post-septal canal joins the nephridial mass, the canal is thrown into fairly tight, somewhat spiraled convolutions (I, Fig. 7). It then winds back and forth along the medial surface, roughly parallel to the antero-posterior axis (II, Fig. 7). Next, it passes to the mid-lateral portion of the mass and is once more tightly convoluted (III, Fig. 7). This condition gives way to a relatively straight length which terminates at the nephridiopore (Fig. 7, NPR).

In addition to affording a three-dimensional view of the path of the canal through

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Key to lettering: BV, blood vessel; CIL, cilia; CM, mass of cilia; EG, eosinophilic granules; EPI, coelomic epithelium; NB, band of nuclei of nephrostome; NC, nephridial canal; NPR, nephridiopore; NST, nephrostome; CP, cytoplasmic processes of nephrostome; PSC, post-septal canal; SEP, intersegmental septum; VSV, ventral segmental blood vessel; I, II, III, first, second, and third regions of the nephridial canal, respectively.

FIGURE 2. General view of a nephridium, transverse section (8 μ, Harris' haematoxylin and eosin; the cavity extending internally from the area of the nephridiopore, NPR, is a longitudinal fold of the body wall; the ventral nerve cord is to the left of the figure and the parapodium is to the lower right).

FIGURE 3. View of nephridium and its associated nephrostome, frontal section (8 μ, Harris' haematoxylin and eosin).

FIGURE 4. Detailed view of nephridial tissue (8 μ, Harris' haematoxylin and eosin).

FIGURE 5. Detailed view of the nephrostome of Figure 3.

FIGURE 6. Detailed view of the nephridiopore, transverse section (8 μ, Harris' haematoxylin and eosin; the large cavity extending toward the upper left of the figure is a longitudinal fold of the body wall).

FIGURE 7. Plaster reconstruction of the nephridial canal, view of the anterior face. (The consecutive numbers indicate the course of the canal; section I, 1–18; section II, 19–30; section III, 31–55.)

the nephridium, the reconstruction shows three regions which merge gradually into one another. After the narrow post-septal canal joins the nephridium, the canal becomes slightly enlarged through the first region of convolution (I, Fig. 7). The canal is then further enlarged to its maximal diameter as it passes to the medial surface (II, Fig. 7). It becomes narrowed in the second series of tight convolutions (III, Fig. 7), and it is at its minimal diameter as it passes to the nephridiopore. This condition is reflected to some extent in Figure 8, which is a graph of the inner diameter along the length of the canal (a measurement of the outer diameter, which would show the thickness of the canal wall, was not possible, due to the poor definition of the cells of the wall). It is of interest to mention the regions of the tubule within the nephridium, as determined by Goodrich and Krishnan, although such differences may well be due to observations of different species. In *N. diversicolor,* Goodrich (1893) found a much convoluted portion with few cilia, into which the post-septal canal led. The next region was very narrow, and the cilia here were confined to one side of the canal. The last section was short, less convoluted, moderately wide, and without cilia. In *N. chilkaensis,* Krishnan (1952) found that the first portion of the canal, as it enters the nephridium, is convoluted and ciliated. The next portion is wider, without cilia, and longer than the preceding section.

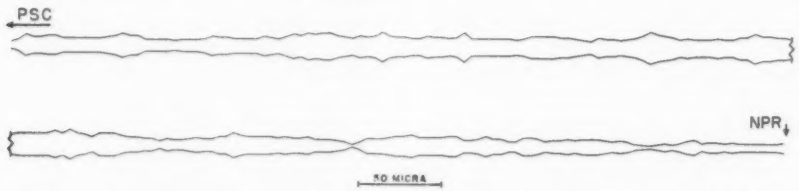

FIGURE 8. Graphic representation of the inner diameter of that part of the nephridial canal within the nephridial mass (reconstructed by measuring the shortest diameter of elliptical sections of the nephridial canal).

This latter portion gives way to a canal which leaves the body of the nephridium and terminates at the nephridiopore.

The mean diameter of the nephridial canal upon which the reconstruction (Fig. 7) and the graph (Fig. 8) were based, was 24 μ, and the over-all length of the canal within the nephridial mass (not including the nephrostome or the post-septal canal) was approximately 1.7 mm.

The author wishes to acknowledge advice, criticisms, and suggestions from Dr. Ralph I. Smith and Dr. Kenneth B. DeOme of this Department, and from Dr. Willard D. Hartman, of the Peabody Museum, Yale University.

SUMMARY

1. The morphology of the nephridia of *Nereis vexillosa* Grube is described.

2. Comparisons are made with the morphology of the nephridia of certain other nereids and differences are noted. Chief among these are, in *N. vexillosa:*

a. that ciliation extends along the whole length of the nephridial canal, with the exception of a short region just preceding the nephridiopore;

b. that three general regions of the nephridial canal are noted, on the basis of the diameter and the amount of convolution;

c. that the wall of the nephridiopore appears to be inserted on the invaginated surface cuticle.

3. A reconstruction of the nephridial canal is presented in which the course of the canal is readily seen.

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STUDIES ON THE ISOLATED ISLET TISSUE OF FISH.[1] II. THE EFFECT OF ELECTROLYTES AND OTHER FACTORS ON THE OXYGEN UPTAKE OF PANCREATIC ISLET SLICES OF TOADFISH, USING THE CARTESIAN DIVER MICRORESPIROMETER

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We have undertaken a detailed characterization of the metabolism of islet tissue because we believe that these studies may provide the basis for understanding the factors which control insulin synthesis and the mechanism by which alloxan and other toxic agents selectively kill the insulin-producing cells (Lazarow, 1949). In approaching this problem we have found it convenient to use the toadfish as an experimental animal. Whereas in mammals the islet tissue is distributed throughout the pancreas in a million or more individual islets of Langerhans totaling only 1% of the pancreatic mass, in the toadfish the islet cells are segregated into one or more discrete bodies which are located in the mesentery and which are called the principal islets (Diamare, 1899; Rennie, 1905). The pancreatic acinar tissue in the toadfish does not form a definite organ; rather, it is diffusely scattered throughout the mesentery, along the bile ducts, and within the liver.

In a previous study (Lazarow and Cooperstein, 1951) we have measured the activity of certain specific enzymes (cytochrome oxidase and succinic dehydrogenase) in normal toadfish islet tissue homogenates. However, in order to characterize the over-all metabolic pathways in islet tissue, it is important to measure the endogenous oxygen uptake as well as that following the addition of specific exogenous substrates. We have therefore studied the endogenous respiration of islet tissue slices and in the present paper we are reporting the effect of varying pH, tonicity, electrolyte composition, and other factors. By means of these studies we have been able to define the conditions under which maximal respiration of the islet tissue slices occurs. This should provide a base-line for subsequent work, which will include a study of (a) the effect of various substrates known to play a role in intermediary metabolism, (b) the effect of various inhibitors and, (c) the effect of hormones and other agents which influence the blood sugar level and/or insulin secretion.

Methods

Mature toadfish, *Opsanus tau*, weighing 200 to 600 gm. were used. During the summer months the animals were kept in a running sea water tank, and they were

[1] Aided by a grant from the National Science Foundation (G-1928) and from the National Institute for Arthritis and Metabolic Diseases of the U. S. Public Health Service (#A-720).

killed within several weeks of the time that they were caught. During the fall and winter months the toadfish were kept in a live car for varying periods of time (1–6 months) after which they were shipped inland by air express from the Marine Biological Laboratory, Woods Hole, Massachusetts. The fish were stored for periods up to one week in an aerated sea water tank (30-gallon crock). The temperature of the sea water was maintained below 20° C. by circulating cold water through the inside of submerged lead coils.

The cartesian divers used were of the cylindrical type without a bulb and they were silicone-treated prior to use. Before the fish were killed, the divers were filled with the various liquid media to be studied; they were completely filled except for a 1–2 mm. air bubble at the very bottom of the diver. The divers were then cooled to 0° C. by placing them in a cooling block. During the intervals between manipulative procedures, the divers were stored in this cold block. After the toadfish were decapitated, the islets were dissected from the mesentery and placed on a piece of Parafilm. The connective tissue capsule surrounding the islet was removed. A petri dish cover, containing a piece of wet filter paper, was placed over the tissue; this served as a moist chamber. The razor blade used for cutting the islet slices was previously cleaned with sodium hydroxide (to remove all traces of oil), thoroughly rinsed in tap water, and finally washed in distilled water. In general the islet obtained from one toadfish was used for each day's experiment; it was cut into eight pieces, each weighing approximately 0.1 to 0.2 mg. wet weight. A slice of islet tissue was then placed at the lower air-liquid meniscus in each of eight divers with the aid of a fine stainless steel needle. Most of the liquid medium was then removed from the diver using a micro pipette; however, a cylindrical segment of medium, about 1 mm. in length, was left behind to form the tissue seal containing the islet slice. With the aid of the cartesian diver filler (Lazarow, 1950), the neck seals of sodium hydroxide, oil and flotation medium were successively placed, in the stated order, above the tissue sample. The divers were then transferred to a thermostatically controlled water bath maintained at 25° C. ± 0.01° C. The pressure was measured using a Wallace Tiernan gauge (Belleville, New Jersey) which was initially suggested and used by Claff (personal communication, 1948). This gauge was calibrated in millimeters of Brodie's solution. A compensating device (Lazarow and Bloomfield, unpublished) connected to the outer chamber of the gauge was used to minimize the effect of changes in barometric pressure. An initial reading was taken after a 20-minute equilibration period. Subsequent readings were taken at 20-minute intervals during the next hour. Thermobar divers usually showed a pressure change of less than 4 mm. per hour, whereas experimental divers showed a change up to 100 mm. per hour. At the end of the experiment the divers were removed from the water bath and the sodium hydroxide, oil and flotation medium seals were removed. The tissue slice plus the tissue seal were transferred from the diver to a smaller model of the conical-tipped micro homogenizer (Lazarow and Portis, 1951), using a capillary pipette. The tissue was homogenized in 100 μl. of water and three 25-μl. aliquots were removed for protein estimation. The protein was determined by a modification of the method of Lowry, Rosenbrough, Farr and Randall (1951). Twenty-five μl. of the sample were mixed with 250 μl. of the protein reagent; the mixture was allowed to stand at 45° C. for ten minutes and, at the end of this time, 25 μl. of the diluted phenol reagent were added. The absorption was read after 15 minutes at room temperature in the Beckman spectro-

photometer at 700 mμ. The amount of protein in the sample was determined by comparing the extinction with that obtained using a standard serum albumin solution. The metabolic activity was expressed as millimicroliters (mμl.) of oxygen taken up per microgram of protein per hour.

Results

At the time this study was begun the osmotic pressure of toadfish blood (sample obtained from the gill) had been determined and found to be equivalent to 0.19 *M* NaCl (Green, personal communication). In the first group of ex-

Table I

Effect of phosphate buffer on islet tissue respiration

Series	mμl. O_2/μg. protein/hr. in saline (0.19 *M* NaCl)			mμl. O_2/μg. protein/hr. in saline-phosphate (0.033 *M* Na_2HPO_4–KH_2PO_4, pH 7.4 + 0.144 *M* NaCl)			p**
	No. determinations	Aver.	σ*	No. determinations	Aver.	σ*	
1	11	1.42	0.80	10	2.24	0.72	.012
2	17	0.78	0.42	14	1.97	0.89	<.001
3	17	1.54	0.41	17	2.20	0.70	.001
4	—	—	—	15	2.62	1.00	—
5	—	—	—	8	2.63	0.84	—
6	—	—	—	9	2.26	1.00	—
7	—	—	—	19	2.08	0.99	—
8	—	—	—	24	2.12	0.95	—
9	—	—	—	16	2.72	0.78	—
10	—	—	—	15	2.32	0.97	—
11	—	—	—	14	2.14	0.79	—
Aver.	45	1.22†	—	161	2.28†	0.37†	

* $\sqrt{\frac{\Sigma \text{ (deviations from mean)}^2}{N}}$.

** Calculated from the formula $\sqrt{\frac{\sigma_1^2}{N_1} + \frac{\sigma_2^2}{N_2}}$. Difference

† Each series taken as one figure.

periments shown in Table I, the respiration of islet tissue in 0.19 *M* NaCl was compared with that observed in a saline-phosphate buffer mixture (containing 0.144 *M* saline and 0.033 *M* Na_2HPO_4–KH_2PO_4 buffer, pH 7.4). In three series of experiments the oxygen uptake of islet tissue in the saline-phosphate mixture was 43 to 153% greater than that observed in 0.19 *M* saline. The *p* values indicated that the differences were all highly significant. Table I also shows that in a group of 11 experiments in which the saline-phosphate mixture was used, there was excellent reproducibility in the average oxygen uptake for the individual series. On the other hand, in the three series of experiments in which 0.19 *M* NaCl was

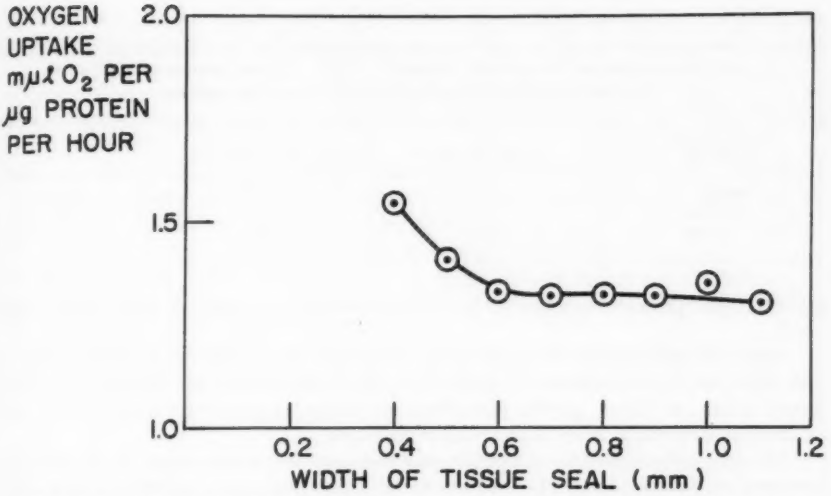

FIGURE 1. Effect of cartesian diver tissue seal width on the respiration of islet slices suspended in 0.19 *M* NaCl. All of the points on the graph in which the tissue seal width was 0.8 mm. or less represent an average of 19 or more individual determinations; the other points represent the average of 5 to 13 individual determinations.

used, the reproducibility was not as good as that observed in the saline-phosphate mixture. A possible explanation of this is shown in Figure 1. The volume of the tissue seal varied considerably from experiment to experiment and in Figure 1 the oxygen uptake of islet slices in 0.19 *M* saline was plotted against the volume of tissue seal in the diver, *i.e.*, the volume of fluid medium surrounding the tissue slice. The metabolic activity of the islet slices in 0.19 *M* saline decreased as the volume of the tissue seal increased. Moreover, this did not occur when phosphate was used; therefore, this greater metabolic variability in saline may be the result of variable dilution of the phosphate extracted from the tissue. Since phosphate addition stimulated the respiration of islet, one would expect that the oxygen uptake would be greater in the divers with small tissue seals.

The oxygen uptake of the islet tissue slices, when expressed as mμl. of oxygen per

TABLE II

Effect of different proportions of Na^+ to K^+ on islet tissue respiration. All media contained 0.033 M phosphate buffer pH 7.4 + 0.144 M NaCl or KCl. The proportions of Na^+ to K^+ were changed by varying the buffer and saline cations

Ratio Na^+/K^+ in medium	No. determinations	Aver. mμl. O_2/μg. protein/hr.	σ
100/0	16	2.77	1.26
96.5/3.5*	15	2.62	1.00

* Saline-PO_4 mixture as described in Table I.

TABLE III

Effect of different proportions of Na^+ to K^+ on islet tissue respiration. All media contained 0.033 M phosphate buffer pH 7.4 + 0.144 M NaCl or KCl. The proportions of Na^+ to K^+ were changed by varying the buffer and saline cations

Ratio Na^+/K^+ in medium	No. determinations	Aver. mμl. O_2/μg. protein/hr.	σ
100/0	16	2.77	1.26
80/20	16	3.24	1.46
0/100	17	3.26	1.33

μg. of tissue protein, appears to be independent of the size of islet tissue slice used.

Since the stimulation observed in the saline-phosphate buffer medium could be due either to (a) the addition of potassium ion (contained in the buffer), (b) buffering action, or (c) a specific phosphate ion effect, experiments were carried out in order to determine which factor was responsible.

The effect of varying the potassium and sodium ion concentrations of the medium is shown in Tables II and III. In the usual saline-phosphate buffer medium there are three and one half parts of potassium to ninety-six and one half parts of sodium. When an all-sodium phosphate buffer was used (prepared by mixing sodium monobasic and sodium dibasic phosphates), there was no change in metabolic activity of the islet slices. When higher potassium ion concentrations were used (Table III), the activity was about 20% higher than that observed with an all-sodium medium. However, analysis of these results showed that this difference was not statistically significant. Since the addition of phosphate buffer increased the metabolic activity of islet slices by 43 to 153%, it may be concluded that this stimulation is not due to the addition of the potassium ion contained in the buffer.

The effect of pH. Various saline-phosphate-buffer mixtures were prepared in which the pH of the phosphate buffer was varied. Table IV shows that there were no significant differences in the metabolic activity of islet slices when the pH was varied from 6.2 to 8.0; the p values were all greater than 0.3. This finding is in keeping with the studies previously reported by other investigators (Elliott and Birmingham, 1949) who suggest that the internal pH of tissue slices is maintained fairly constant over a wide range of external pH. This finding therefore suggests that the stimulatory effect observed with phosphate buffer addition (Table I) was

TABLE IV

Effect of pH on islet tissue slice respiration. In all cases the medium consisted of 0.144 M NaCl + 0.033 M Na_2HPO_4–KH_2PO_4 buffer

pH of medium	No. of determinations	Aver. mμl. O_2/μg. protein/hr.	σ
6.2	11	2.39	0.67
6.8	14	2.46	0.89
7.4	14	2.14	0.79
8.0	15	2.53	1.25

due to a specific phosphate ion effect rather than to the ability of the phosphate buffer to maintain the pH of the medium.

The effect of buffer type. The metabolic activity of islet slices was studied in phosphate, tris (trishydroxyaminomethane), and veronal buffers at pH 7.4 and the results are shown in Table V. The highest metabolic activity was observed with phosphate buffer. With an equimolar concentration of tris buffer, the oxygen uptake was lower than with phosphate buffer (p value $< .001$), but it was of the same order of magnitude as with 0.19 M saline (Table I). With veronal buffer the metabolic activity was 86% lower than with phosphate and much lower than with 0.19 M saline. Thus veronal buffer inhibits the metabolic activity of islet slices. Since in the presence of 0.033 M tris plus 0.033 M phosphate buffer the O_2 uptake was the same as in phosphate, the low activity in tris buffer was not due to inhibition, but rather to the absence of phosphate.

The effect of phosphate concentration. A large series of experiments were carried out in which varying concentrations of the Na_2HPO_4–KH_2PO_4 buffer (pH 7.4)

TABLE V

Effect of buffer type on islet tissue slice respiration. In all cases the medium contained 0.033 M buffer, pH 7.4; the medium containing both tris and phosphate buffer was 0.033 M with respect to each. Enough NaCl was added to each medium to maintain a tonicity equivalent to 0.19 M NaCl

Buffer in medium	Series I			Series II		
	No. determinations	Aver. mμl. O_2/μg. protein/hr.	σ	No. determinations	Aver. mμl. O_2/μg. protein/hr.	σ
Na_2HPO_4–KH_2PO_4	15	2.32	0.97	19	2.08	0.99
Tris	14	1.29	0.64	18	1.60	0.47
Veronal	6	0.33	0.13	—	—	—
Tris Na_2HPO_4–KH_2PO_4	—	—	—	19	2.02	0.52

were added and the tonicity of the medium was maintained equivalent to 0.19 M NaCl by adjusting the NaCl concentration. These results are shown in Figure 2. There was a progressive increase in the oxygen uptake as the phosphate ion concentration was increased. Maximal stimulation of metabolic activity was observed at a phosphate ion concentration of 0.066 M. The stimulation observed in the presence of 0.066 M phosphate was highly significant. A comparison of the activity in 0.066 M phosphate with that observed in the absence of phosphate, or in the presence of 0.002 M or 0.008 M phosphate, gave a p value < 0.001. The difference between 0.066 M and 0.033 M phosphate is also probably significant (p value $= 0.057$). At a phosphate ion concentration greater than 0.066 M there was no further stimulation; in fact the value at 0.136 M is actually 5% lower than at 0.066M.[2] This difference, however, is not significant ($p = 0.6$).

[2] Later studies on the effect of tonicity showed that at the high concentrations of phosphate one would have expected a large decrease in metabolic activity due to the increasing tonicity of the medium. In the presence of 0.136 M phosphate, this decrease should have been much larger than the 5% actually observed. The fact that a decrease of only 5% was observed using 0.136 M phosphate suggests that the higher concentration of phosphate is effectively stimulating the metabolic activity but that this stimulation is masked by inhibitory effects of increasing

The effect of tonicity. In order to study the effect of tonicity, the oxygen uptake of islet slices was measured in media of varying salt concentrations. However, since the phosphate buffer concentration that gives maximal stimulation (0.066 *M*) has a tonicity equivalent to 0.093 *M* NaCl, it was necessary to reduce the phosphate ion to a suboptimal value if lower tonicities were to be used. Therefore additional

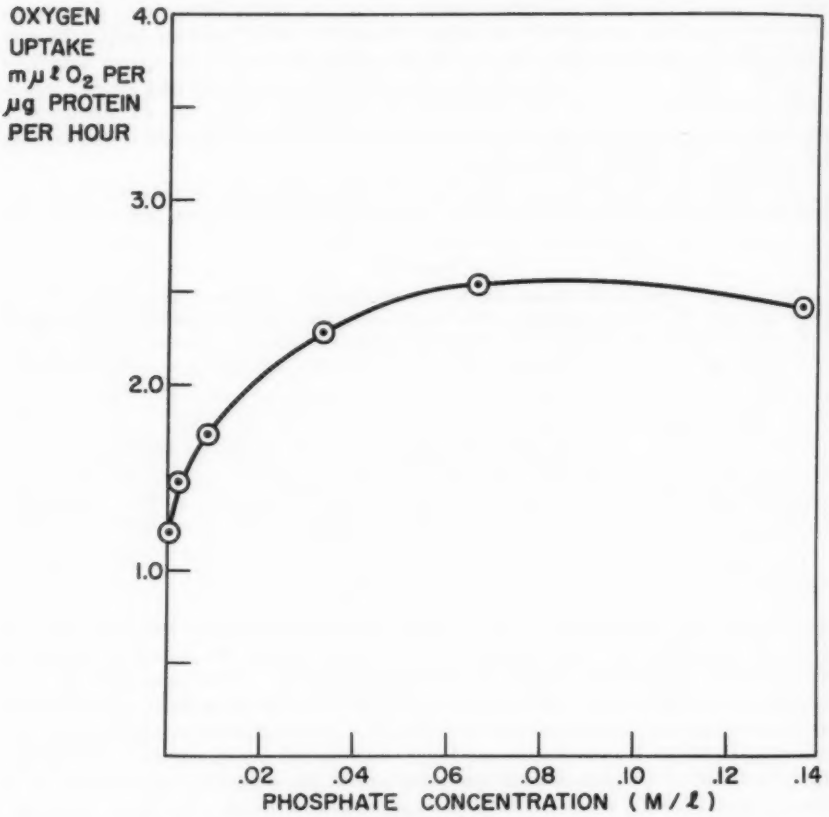

FIGURE 2. Effect of phosphate ion concentration on the respiration of toadfish islet slices. The tonicity of the medium was maintained equivalent to that of 0.19 *M* NaCl; pH = 7.4. Each of the points represents the average of 32 to 161 individual determinations.

studies were carried out with lower phosphate concentrations. Where necessary the tonicity was adjusted by adding NaCl. Table VI shows that the oxygen uptake increased with decreasing tonicity. When the tonicity of the medium was equivalent to 0.093 *M* NaCl, the metabolic activity of islet slices was 60% greater ($p = 0.006$)

tonicity. Thus the appearance of a maximum at a phosphate concentration of 0.066 *M* may be more apparent than real.

than that observed when the tonicity was equivalent to 0.19 *M* NaCl. When the tonicity was decreased further, the oxygen uptake diminished. Furthermore it should be noted that at a tonicity equivalent to 0.093 *M* NaCl, a progressive decrease in the phosphate ion concentration from 0.066 to 0.033 to 0.017 *M* gave a slight but progressive decrease in metabolic activity of the islet slices. This decrease is of the order of magnitude that would be expected from the phosphate curve (Fig. 2).

In order to explore more fully the effect of the tonicity of the medium, a series of experiments were carried out in which the phosphate ion concentration was maintained at 0.033 *M* (a slightly sub-optimal phosphate level) and in which the tonicity was varied between a sodium chloride equivalent of 0.048 *M* and 0.50 *M*. The results are shown in Figure 3. The maximum activity was observed at a tonicity equivalent to 0.075 *M* NaCl. At higher or lower tonicities the oxygen uptake was less than this optimal value. On statistical analysis the differences between the oxygen uptake at a tonicity equivalent to 0.075 *M* NaCl and those at the following

TABLE VI

Effect of varying tonicity on islet tissue slice respiration in phosphate buffer. The media contained varying concentrations of Na_2HPO_4–KH_2PO_4 buffer, pH 7.4; the proper tonicity was attained by adding the appropriate concentration of NaCl

PO_4 concentration (*M*)	Tonicity of medium (equivalent NaCl concentration) *M*/*l*	No. determinations	Average mμl. O_2/μg. protein/hr.	σ
0.066	0.76	8	1.84	0.93
0.066	0.380	8	2.37	0.92
0.066	0.190	8	2.68	1.18
0.066	0.093	8	4.43	0.91
0.033	0.190	8	2.62	0.84
0.033	0.093	8	4.10	1.28
0.033	0.047	8	3.16	1.05
0.017	0.093	8	3.35	0.93

tonicities were found to be significant: 0.047 *M* ($p = 0.036$), 0.147 *M* ($p = 0.036$), 0.190 *M* ($p = 0.03$). Although the differences between the oxygen uptake at 0.075 *M* and those at the other tonicity values were not significant on statistical analysis, these values nevertheless fall into a smooth curve (Fig. 3). Although a more precise localization of the maximum could be determined if a larger number of studies were carried out, there is little doubt that maximum stimulation occurs at or near a tonicity equivalent to 0.075 *M* NaCl.

Since 0.066 *M* phosphate buffer has a tonicity equivalent to 0.093 *M* NaCl, it is obvious that one cannot simultaneously achieve the conditions for both optimal phosphate and optimal tonicity. In order to achieve the most effective compromise, we have compared the respiration of islet tissue slices at pH 7.4 and at optimal phosphate sub-optimal tonicity (0.066 *M* Na_2HPO_4–KH_2PO_4 buffer, tonicity equivalent to 0.093 *M* NaCl) with that obtained at sub-optimal phosphate-optimal tonicity (0.054 *M* Na_2HPO_4–KH_2PO_4 buffer, tonicity equivalent to 0.075 *M* NaCl). The oxygen uptake in 0.054 *M* phosphate buffer was 3.54 mμl. per μg. protein per hour:

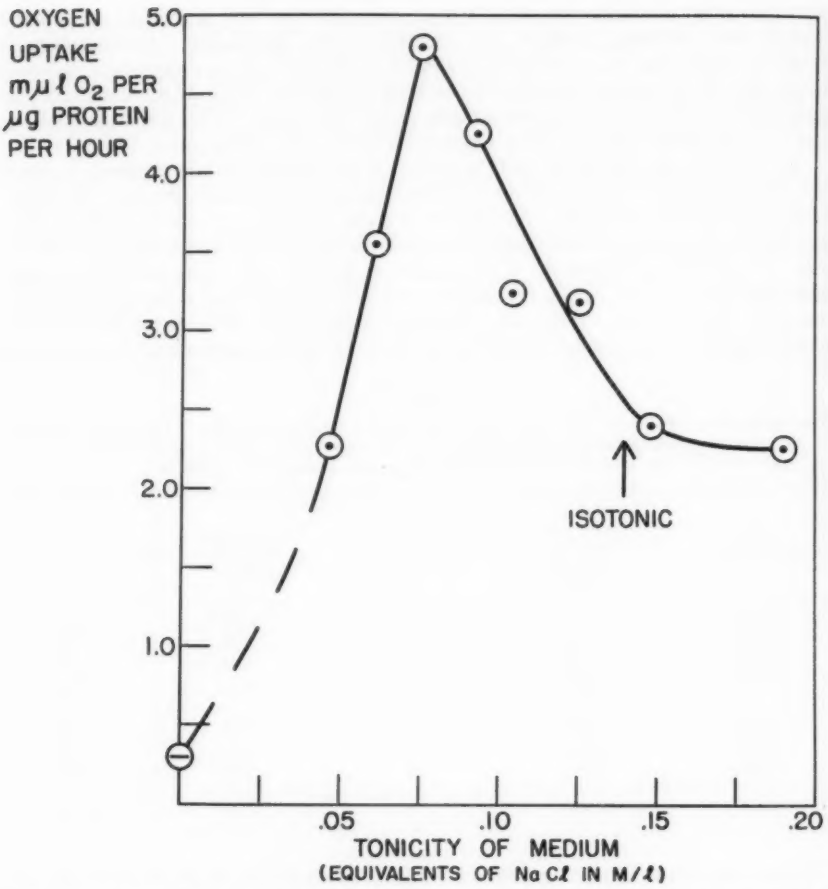

FIGURE 3. Effect of tonicity of the medium on the respiration of islet slices suspended in 0.033 *M* Na_2HPO_4—KH_2PO_4 buffer, pH 7.4. Each of the points represents the average of 8 to 10 individual determinations. The measurements at zero tonicity were carried out in distilled water and therefore this point represents the respiration in the absence of phosphate.

this was 5% greater than the activity in 0.066 *M* phosphate buffer. Although this difference is slight, it does agree with the value that would be expected on the basis of data shown in Figures 2 and 3. A decrease in the phosphate concentration from 0.006 *M* to 0.054 *M* would bring about a 3% decrease in the metabolic activity (Fig. 2), whereas a change in the tonicity of the medium from a sodium chloride equivalent of 0.093 *M* to 0.075 *M* would bring about a 13% increase in the metabolic activity. Therefore in the subsequent studies we used a 0.054 *M* Na_2HPO_4–KH_2PO_4 buffer, pH 7.4, which has a tonicity equivalent to 0.075 *M* NaCl.

The effect of calcium. Media containing a constant amount of phosphate buffer (0.054 *M*) and varying amounts of calcium chloride ranging from 0.002 *M* to 0.00005 *M* were prepared. Since the final tonicity of these solutions ranged from a sodium chloride equivalent of 0.075 *M* to 0.077 *M*, the expected changes in metabolic activity as a consequence of tonicity changes would be insignificant. The addition of calcium ion at a concentration of 0.002 *M* produced a 49% inhibition ($p < 0.001$) of oxygen uptake; at 0.001 *M* it produced a 25% inhibition ($p = 0.02$) of oxygen uptake; at concentrations of 0.0005 *M* and 0.00005 *M* it did not affect the metabolic activity.

The effect of magnesium. The effect of magnesium ion addition was tested both in the presence and absence of added calcium. Magnesium, in concentrations ranging from 0.01 *M* to 0.002 *M*, did not affect the metabolism of the islet tissue slices either in the absence or presence of calcium chloride (1×10^{-4} *M* $CaCl_2$).

The effect of trace metals. A mixture of trace metal salts, constituting the minimal trace element requirements for Neurospora growth (Beadle, 1945), was added to 0.054 *M* phosphate buffer. The final concentrations of the trace elements per liter of medium were: boron 0.01 mg., molybdenum 0.02 mg., iron 0.2 mg., manganese 0.02 mg., and zinc 2.0 mg. These metals were added as the following salts: $Na_2B_4O_7$, $(NH_4)_2MoO_4$, $FeCl_3$, $MnCl_2$, and $ZnCl_2$. In addition, two other media were prepared in which the concentrations of the trace metals were 10 times and 100 times greater, respectively, than those listed above. The addition of the above trace metals at the minimal concentration did not affect the respiration of the islet tissue slices. When the concentration of each trace metal was increased 10-fold there was a 15% inhibition ($p = 0.44$) of the oxygen uptake; when increased 100-fold, there was a 30% inhibition ($p = 0.05$).

The effect of pyrophosphate. The respiration of islet slices was studied in a medium containing 0.005 *M* pyrophosphate plus 0.054 *M* phosphate buffer. The added pyrophosphate can act as a chelating agent and thus remove trace metal ions. Its addition, however, did not affect the respiration of islet tissue slices.

The effect of serum. Samples of toadfish blood were drawn from the gill by venipuncture and the serum separated by centrifugation. The oxygen uptake of islet slices in serum was compared with that in 0.054 *M* phosphate buffer and found to be 34% lower ($p = 0.007$). On the other hand, when the serum was previously dialyzed against three liters of 0.054 *M* phosphate buffer for 18 hours at 0° C., the oxygen uptake almost equaled (95%) that in the 0.054 *M* buffer. Part of this difference may be due to a phosphate ion effect. However, since the tonicity of toadfish serum is considerably greater than the tonicity of 0.054 *M* phosphate (equivalent to 0.075 *M* NaCl), the low values obtained in serum, and the higher values obtained in dialyzed serum, can in part be due to tonicity differences. From the results obtained in Figure 3 one would expect that an increase in tonicity to that found in serum would produce a 50% decrease in the oxygen uptake, whereas a 35% decrease was actually found. These results suggest that the addition of serum protein *per se* does not materially affect the respiration of islet slices.

Discussion

It should be noted that maximal stimulation of islet respiration was observed at a tonicity equivalent to 0.075 *M* NaCl; this is considerably lower than the tonicity

of toadfish blood.[3] Similar stimulation of the oxygen uptake of brain homogenates when suspended in hypotonic media has been reported by Elliott and Libet (1942). The activity of the succinic oxidase system is likewise increased in hypotonic media and this stimulation is believed to be a direct effect on the enzyme complex (Tyler, 1954).

The stimulation of toadfish islet metabolism by phosphate is of interest. The addition of phosphate increases the oxygen uptake of brain homogenates (Elliott and Libet, 1942); it also increases the activity of certain isolated enzyme systems (Kearney, Singer and Zastrow, 1955; *cf.* Koeppe, Boyer and Stulberg, 1956). It has been suggested that it may also play a role in the control of respiration (*cf.* Lardy and Wellman, 1952). It would therefore be of interest to determine whether the oxygen uptake of slices of other toadfish tissues is similarly stimulated by phosphate addition, or if this stimulation is limited to islet tissue. Since islet tissue contains large amounts of zinc (Okamoto, 1942), and since high concentrations of the trace metal ions inhibited the oxygen uptake of the islet tissue, the phosphate stimulation could be the result of zinc chelation. It would therefore be of interest to see if pyrophosphate or other chelating agents can substitute for phosphate.

Islet tissue contains about 11%[4] protein and therefore the oxygen uptake of islet tissue slices (3.5 mμl. of oxygen consumed per μg. of protein per hour) would be equivalent to 0.39 cc. per gram of tissue (wet weight) per hour. This observed value is about equal to the reported value for brain brei and greater than that reported for liver slices. Vernberg (1954) found that the oxygen uptake of toadfish brain brei was equal to 0.41 cc./gm./hr.; the Q_{O_2} of toadfish liver slices was equal to 0.27 cc./gm./hr. The Q_{O_2} of the toadfish islet tissue is about 10 times greater than the oxygen utilization by the intact animal. Hall (1929) has reported that the oxygen consumed by the toadfish varies directly with the oxygen tension (between a partial pressure of 0 and 115 mm. of oxygen). At the atmospheric oxygen content he found that the toadfish utilized 0.038 cc. of oxygen per gram of fish per hour. Thus the toadfish islet tissue is very active metabolically compared to the fish as a whole and to the other tissues.

Summary

1. The metabolic activity of toadfish islet slices was measured in a cartesian diver microrespirometer under varying experimental conditions. The effects of pH, specific electrolytes, tonicity, trace metals, and protein addition were studied.

2. The metabolic activity was not affected by varying the pH of the medium between 6.2 and 8.0. The addition of phosphate ion stimulated the respiration. The maximum stimulation was observed when the external medium contained 0.066 *M* phosphate.

3. The respiration of islet slices was increased when the tonicity of the suspending media was reduced; optimal respiration was observed in a hypotonic me-

[3] Green and Hoffman (1953) have measured the osmotic pressure of blood samples obtained from the heart and found them to be equivalent to 0.14 *M* NaCl; the osmotic pressure of blood samples obtained from the gill were equivalent to 0.19 *M* NaCl. These authors consider the tonicity values of the heart blood samples to be the more accurate, for the gill blood samples may have been contaminated with sea water.

[4] The protein content of 5 samples of islet tissue was measured and found to contain 11.2% protein.

dium with a tonicity equivalent to 0.075 *M* NaCl; this corresponds to a phosphate buffer concentration of 0.054 *M*. This is slightly sub-optimal with respect to phosphate ion concentration. Since the metabolic activity of islet tissue slices suspended in 0.054 *M* phosphate buffer (optimal tonicity but sub-optimal phosphate) is slightly greater than in 0.066 *M* phosphate buffer (optimal phosphate but sub-optimal tonicity), subsequent studies were carried out using 0.054 *M* phosphate buffer.

4. High concentrations of calcium (0.001–0.002 *M*) inhibited the respiration of islet slices. The addition of serum protein, lower concentrations of calcium ion (0.0005 *M*), magnesium ion (0.0002–0.01 *M*), and small amounts of trace metals (boron, molybdenum, iron, manganese, zinc) did not stimulate the respiration of islet tissue slices.

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EVIDENCE FOR HORMONE-CONTAINING GRANULES IN SINUS GLANDS OF THE FIDDLER CRAB UCA PUGILATOR

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It is known that in neurosecretory systems the products of secretion are stored in axon terminations where they aggregate in particles or granules (Scharrer and Scharrer, 1954; Welsh, 1955).

Some recent electron microscope studies have shown a constancy in the appearance of such structures in the neurohypophysis of different vertebrates (Duncan, 1955, 1956). The size of these granules varies from 0.1 to 0.3 micron, and in the neurohypophysis of the rat they seem to be bounded by a delicate membrane (Palay, 1955).

Through the differential centrifugation technique for isolation of mitochondria and other particles of the cells, Hillarp, Lagersted and Nilson (1953) and Blaschko and Welch (1953) could obtain a fraction of granules which is responsible for 80 to 90% of the total adrenaline and noradrenaline present in the adrenal medulla of cattle. Further, Hillarp and Nilson (1954) and Blaschko, Hagen and Welch (1955), doing physiological experiments with the separated granular fraction, obtained information concerning the nature of the granules containing the catechol amines. Similar results were obtained for the granules containing vasopressin and oxytocin in the posterior pituitary of the rat (Pardoe and Weatherall, 1955). The observations of the several authors, above cited, strongly support the assumption that the granules have a semipermeable membrane of a lipid or lipo-protein nature. The granules, which are stable in isotonic solutions of saline or sucrose, release their hormone when treated by agents which are known to damage biological membranes.

In the invertebrates, especially among insects and crustaceans, some neurosecretory systems are very well known, and the study of the granules in these systems might give valuable information concerning such storage particles. A good structure for these studies is the "sinus gland" of the crustaceans. A sinus gland in each eyestalk is the storage-release organ for several neurohormones of the crustaceans. They are formed by the axon terminations of neurosecretory cells localized in the X-organ, in the brain and in other parts of the central nerve system (Passano, 1951a, 1951b; Bliss and Welsh, 1952; Bliss, Durand and Welsh, 1954). The axon terminations in sinus glands are filled with granules 0.1 to 0.3 micron in diameter (Potter, 1956) which can be seen in living preparations (Passano, 1952).

The aim of the present work was to show that the granules in sinus glands are really the depots of neurosecretory materials and that they behave like similar structures found in the neurosecretory systems of vertebrates.

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Material and Methods

For this purpose the study of one of the chromatophorotropic hormones stored in sinus glands was chosen. According to the usual technique for isolation and preservation of mitochondria (Hogeboom, Schneider and Palade, 1948) the sinus glands were homogenized in isotonic solutions of sucrose. The homogenates before and after several treatments were injected into test animals for an estimation of the activity of the hormone in the different cases.

Preparation of the homogenates

Sinus glands of the fiddler crab, *Uca pugilator,* from Florida, were used in these experiments. With the aid of a dissecting microscope the sinus glands were isolated from the adjacent tissues immediately after cutting the eyestalk of the crabs, and were placed in solutions of cold 1.3 *M* sucrose, which, according to Abramowitz and Abramowitz (1938), is isosmotic with the blood of *Uca*. In each experiment four or more sinus glands were homogenized in one ml. of isotonic sucrose, in the Elvehjem homogenizer for three minutes. After homogenization more sucrose was added according the requirement of the experiment. A part of the homogenate was then kept at 2° C. until the moment of the experiment, and the remainder was submitted to different treatments. Before being injected into the test animals all homogenates were diluted in isotonic sucrose, or sea water to make the same final concentration. All the procedures were carried out in the cold at 2° C. For details, see below.

Assays

The activity of the black chromatophore-dispersing hormone in the different homogenates was tested in isolated legs of *Uca pugnax*. It was observed that in legs of *Uca pugilator* when they are separated from the body, the black chromatophores disperse gradually. Such dispersion may be explained by a direct effect of light on the chromatophores, since legs isolated and kept in sea water in the dark do not show this phenomenon. A direct effect of light on the chromatophores of *Uca pugilator* has been already observed by Brown and collaborators (Brown and Sandeen, 1946, 1948; Brown, Guyselman and Sandeen, 1949). For this reason in the present experiments the legs of *Uca pugnax* which do not show this behavior were used.

Uca pugnax were destalked 24 hours before the experiment so that at the time of the experiment the black chromatophores were in the stage of maximal concentration. The legs were cut off at the level of the ischial segment, and were placed in 5 ml. of sea water in Syracuse dishes. Each leg received an injection of 0.01 ml. of homogenate, and the dispersion of the black chromatophores was observed every ten minutes for one hour. *Uca pugilator* was used for experiments in which the homogenates were tested in the whole animal. In these cases, each animal received 0.1 ml. of homogenate and the stages of the chromatophores were observed for several hours.

Preliminary attempts were made to remove granules from the homogenates by centrifugation.

All the results are presented in graphs according to Hogben and Slome (1931),

where 1 represents maximal concentration of the chromatophores, 5 maximal dispersion, and 2, 3 and 4, intermediate stages.

Results

I. Hormone in granules and in cytoplasm

The homogenate of 4 sinus glands in 1 ml. of 1.3 *M* sucrose was divided; one-half of the suspension received 4.5 ml. of distilled water and was kept at 2° C.; the other half was kept undiluted at the same temperature. After one-half hour, the homogenate in sucrose was diluted with sucrose, and that with added distilled

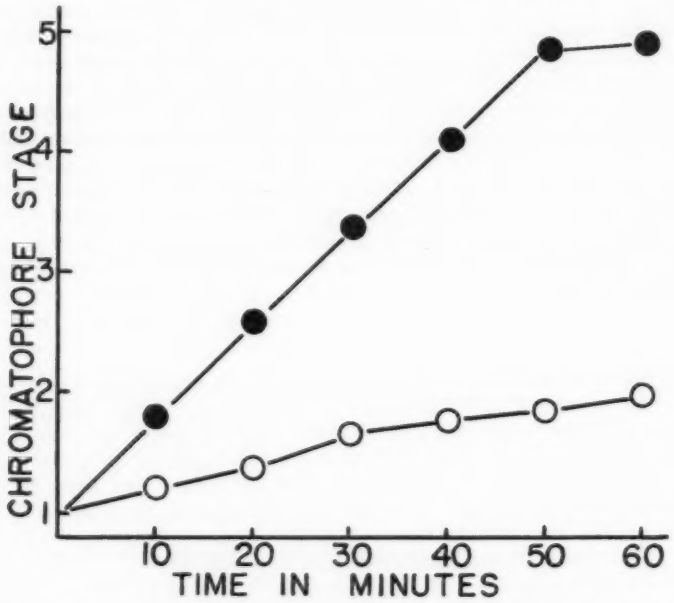

FIGURE 1. Response of the black chromatophores of isolated legs of *Uca pugnax* to injections of homogenates of sinus glands of *Uca pugilator*: ●, homogenates in distilled water; ○, homogenates in 1.3 *M* sucrose. Each point in the graph represents the average of 20 experiments.

water was diluted with sea water to make a final concentration of 0.02 sinus gland per ml. Every time that distilled water was added to the homogenates, the final dilution was made in sea water; these homogenates throughout the paper will be called "homogenates in distilled water," to shorten the explanation.

As one can see in Figure 1, the homogenate in distilled water caused maximal dispersion of the chromatophores in the legs of *Uca*, and that in sucrose exhibited only a small effect. These results show that in isotonic sucrose the black chromatophore-dispersing hormone is present in large part in a state in which it cannot act; whereas in distilled water it seems to be free in solution and able to induce the dis-

persion of the chromatophores. This evidence supports the view that the hormone is contained in granules, which in isotonic sucrose remain intact and in distilled water release the hormone into the solution.

If that were the case it would be possible to separate a fraction of granules containing hormone, using the usual technique of differential centrifugation. To avoid the high density of a medium like 1.3 *M* sucrose, 16 sinus glands were homogenized in one ml. of a mixture of 25% of 1.3 *M* sucrose and 75% of sea water, which has been demonstrated to be as effective in preserving the granules as pure sucrose. The homogenate, after dilution to make 10 ml., was centrifuged at 800 × gravity

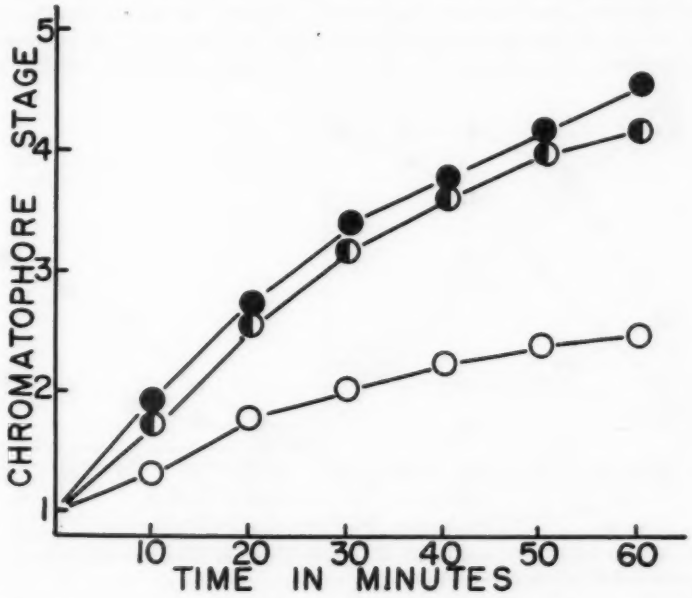

FIGURE 2. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in isotonic sucrose after centrifugation: ●, "supernatant A" (low speed); ◐, "supernatant B" (high speed); ○, "sediment C" (high speed).

for 30 minutes for separation of unbroken cells, nuclei, etc. No visible sediment was observed after this slow-speed centrifugation, so the whole solution was decanted. Part was set aside, as "solution A," and the rest was centrifuged at 20,000 × gravity for 30 minutes. No sediment was observed this time either. The whole solution was decanted carefully and taken as "solution B." Then one ml. of distilled water was added to the centrifuged tube and was stirred and the tube walls were scraped with a spatula. After 15 minutes 9 ml. of sea water were added to make 10 ml., and this solution was called "solution C." Part of the solution A and B was treated with distilled water and all three solutions were finally diluted to the concentration of 0.02 sinus gland per ml. Both solutions A and B showed the same

effect on the chromatophores of legs of *Uca pugnax*. No significant loss of activity was observed in solution B after the high speed centrifugation. However, solution C, the suspension of a presumably invisible sediment, caused a small effect on the chromatophores (Fig. 2). This fact is indicative of some sedimentation of granules and from these results it is not possible to infer how much of the hormone is present in granules and how much is found free in the homogenate. It is probable that for a complete sedimentation a longer and higher-speed centrifugation is necessary.

An indication of the percentage of hormone contained in granules in isotonic sucrose is given by the analysis of the activity of homogenates in isotonic sucrose and distilled water after a series of dilutions. Figure 3 shows the results of injections of 0.01 ml. of homogenates of 2 sinus glands in 1 ml. of 1.3 *M* sucrose and in 1 ml. of distilled water diluted 10, 100 and 1000 times in 1.3 *M* sucrose and sea

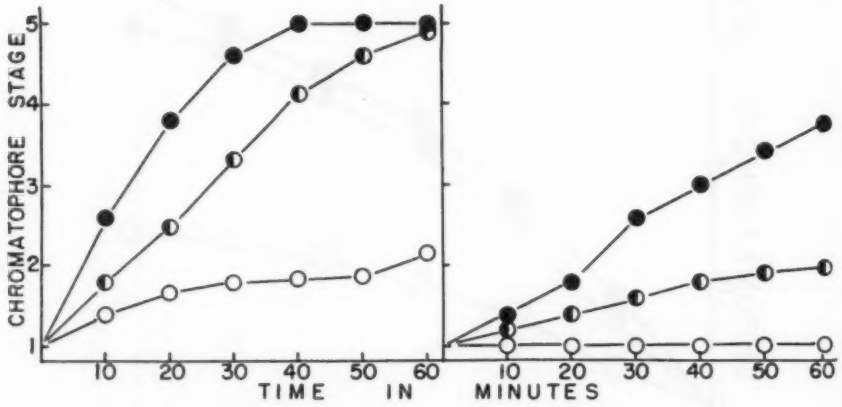

FIGURE 3. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in distilled water (left) and in 1.3 *M* sucrose (right) in different concentrations: ●, 0.2; ◐, 0.02; and ○, 0.002 sinus glands per ml.

water, respectively. It appears that in isotonic solution only less than 10% of the hormone is found free in the suspension, since the effects of the homogenates in sucrose are smaller than those of the homogenates in distilled water, ten times more diluted.

II. Effect of several treatments on the release of the hormone

1. Effect of the tonicity of the medium. From the homogenates of 8 sinus glands in 2 ml. of 1.3 *M* sucrose, 7 samples of 0.25 ml. each were separated. The addition of 9.75 ml. of 0.9, 0.8, 0.7, 0.65, 0.32 *M* sucrose was made to a series of 5 tubes and to a sixth, 4.75 ml. of distilled water were added. After one hour at 2° C., the solutions were diluted in 1.3 *M* sucrose to the final concentration of 0.02 sinus gland per ml. and were tested on isolated legs. Figure 4 illustrates the activity of the hormone in the different solutions. As the tonicity of the medium de-

creases there is a liberation of hormone which, to some extent, is proportional to the concentration of sucrose, from 1.3 to 0.7 *M*. In 0.65 and 0.32 *M* solutions there seems to be a complete release since the activity of the hormone in these two latter concentrations is as great as that of homogenate in distilled water. The action of distilled water after 15 minutes standing is as effective as after 30 minutes. This fact shows that the release in distilled water is rapid. The granules in this respect, like red blood cells and mitochondria under the same conditions, appear to act as osmometers.

2. Effect of different solutions. One group of experiments was performed to determine whether the granules containing the hormone are also stable in isotonic

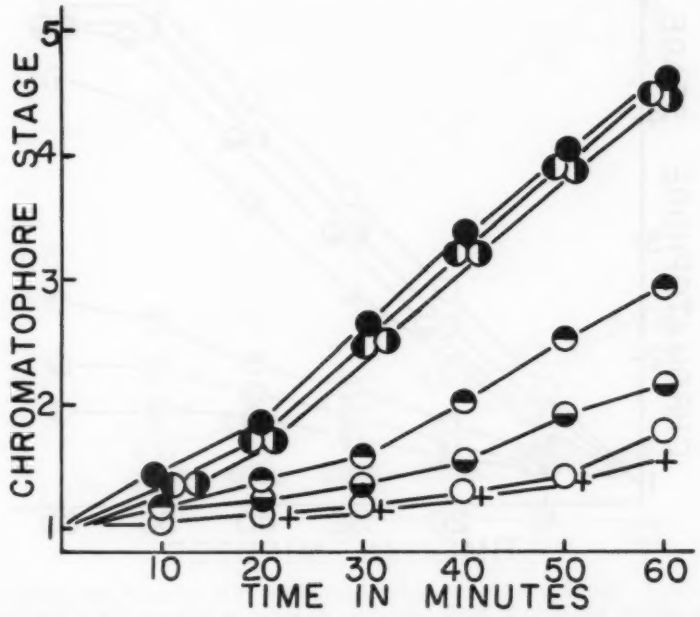

FIGURE 4. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in different concentrations of sucrose solutions: +, 1.3; ○, 0.9; ◒, 0.8; ◓, 0.7; ◑, 0.65; and ◐, 0.32 *M*; ●, homogenate in distilled water.

solutions of electrolytes. Samples of 0.5 ml. of homogenates in 1.3 *M* sucrose were held for 30 minutes at 2° C. with the addition of 4.5 ml. of the following: distilled water, sea water, sodium chloride and potassium chloride. The sodium chloride was either isotonic with sea water (0.54 *M*) or isotonic with 1.3 *M* sucrose (0.78 *M*). Figure 5 shows that sea water and isotonic salt solutions produce a large and rapid release of hormone. This fact indicates that the simple dilution in isotonic electrolyte solutions is sufficient to provoke alterations in the granules very similar to those observed by lowering the tonicity of the medium. However, in electrolyte

solutions to which an equal part or a fourth part of isotonic sucrose is added, the granules remain largely intact. The activity of the hormone in these media (Fig. 5) is comparable to that in isotonic sucrose.

In another group of experiments an effort was made to find the best medium for preservation of the granules. Watanabe and Williams (1953) have shown that 2.5% bovine plasma albumin in isotonic potassium phosphate buffer at pH 7 is a good medium to preserve mitochondria of insect muscles. In the following experiments, besides the 1.3 *M* sucrose, a mixture of 25% 1.3 *M* sucrose and 75% sea water was also used, as well as 2.5% bovine plasma albumin in 0.54 *M* potassium

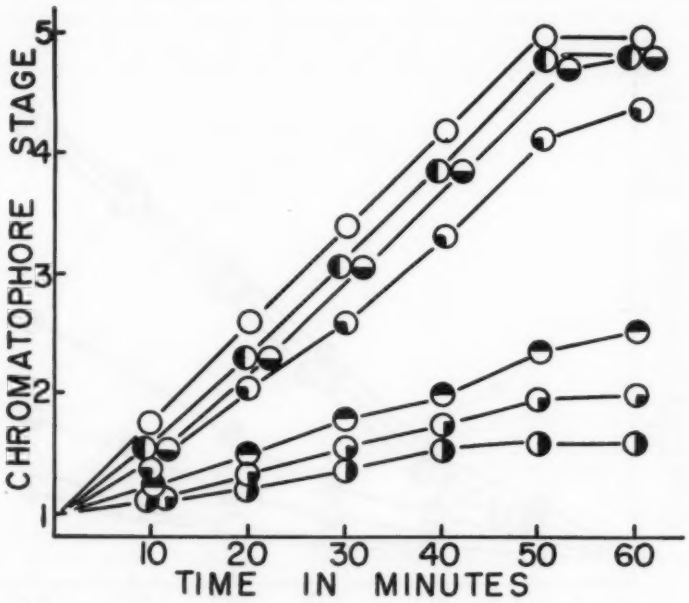

FIGURE 5. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in different solutions: ○, distilled water; ◐, sea water; ◒, 0.78 and 0.54 *M* NaCl; ◔, 0.54 *M* KCl; ◓, ◔, and ◑, NaCl, KCl and sea water in a mixture with 25% of 1.3 *M* sucrose.

phosphate at pH 7. The homogenates of sinus glands in these three media were kept at 2° C. and at different times were diluted in 1.3 *M* sucrose and assayed using legs of *Uca* (Fig. 6). After 6 hours of incubation in these media, the activity of the black chromatophore-dispersing hormone is insignificant, and after 24 hours only a slight effect was observed. That the hormone was preserved in the granules was shown by the following procedure. After 24 hours the homogenates were heated for 5 minutes in boiling water and diluted in sea water. After this treatment all the solutions produced a maximal dispersion of the chromatophores, comparable to that caused by homogenates in distilled water. Thus, the three different media used seem to be equally efficient in keeping the granules intact.

3. Effect of heat, and of freezing and thawing. A release of hormone from the granules was observed when homogenates of sinus glands in isotonic sucrose were kept at room temperature for several hours. However, homogenates in 1.3 *M* sucrose when heated for 5 minutes at 70° C. or in boiling water showed only a slightly greater activity than the original homogenate without this treatment (Fig. 7).

Freezing at − 10° C. and thawing to room temperature three times in succession was more effective than heating, but even so, the release of the hormone was not the same as when homogenate was merely diluted in distilled water (Fig. 7).

4. Effect of detergents and digitonin. Detergents and digitonin did not give a complete release of hormone from the granules. Samples of 0.25 ml. of homoge-

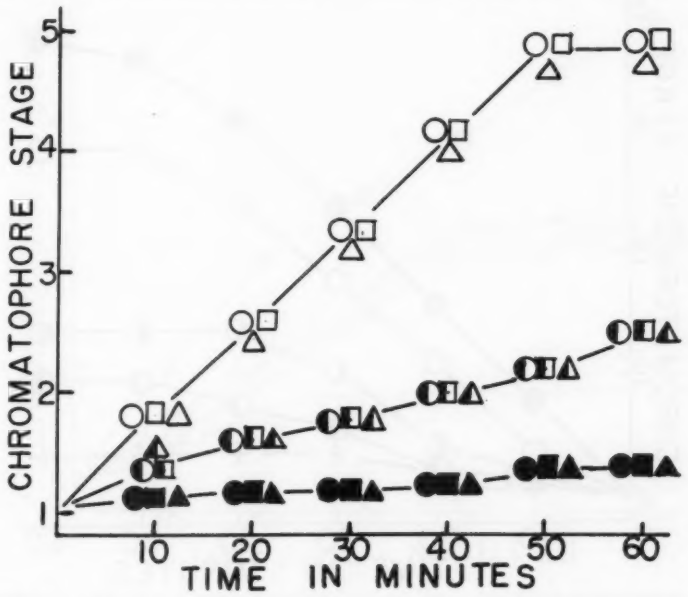

FIGURE 6. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in different media. Circles, homogenate in 5% bovine plasma albumin in 0.54 *M* potassium phosphate buffer; squares, homogenate in 1.3 *M* sucrose; triangles, homogenate in 25% 1.3 *M* sucrose plus 75% sea water. ●, ■, ▲, homogenates kept 6 hours and ◐, ◧, ◭, 24 hours at 2° C.; ○, □, △, after being kept 24 hours at 2° C., the homogenates were heated and diluted in sea water.

nates in 1.3 *M* sucrose were maintained for one hour at 2° C. with 1.75 ml. of 10^{-3} *M* concentration of the following substances: sodium lauryl sulfonate (Duponol); sodium desoxycholate, saponin and digitonin, in 1.3 *M* sucrose. After the required dilution of the homogenates for the bio-assays, the concentration of the detergents and digitonin was 10^{-5} *M*. When control legs of *Uca* or the whole control animal received injections of the detergents and digitonin in such concentration, no effect on the chromatophores was observed. Therefore, the dispersion following the injections of homogenates in sucrose plus detergents is attributed to the hormone present in the solutions.

The detergents employed and digitonin provoked only a partial release of hormone (Fig. 8). Part of the homogenate plus desoxycholate after one hour of incubation was heated and diluted in sea water, and greater activity was seen after this treatment.

III. Inactivation of the hormone

In some experiments the homogenates of sinus glands in distilled water were injected into the whole crab (*Uca pugilator*). Figure 9 shows the degree and the

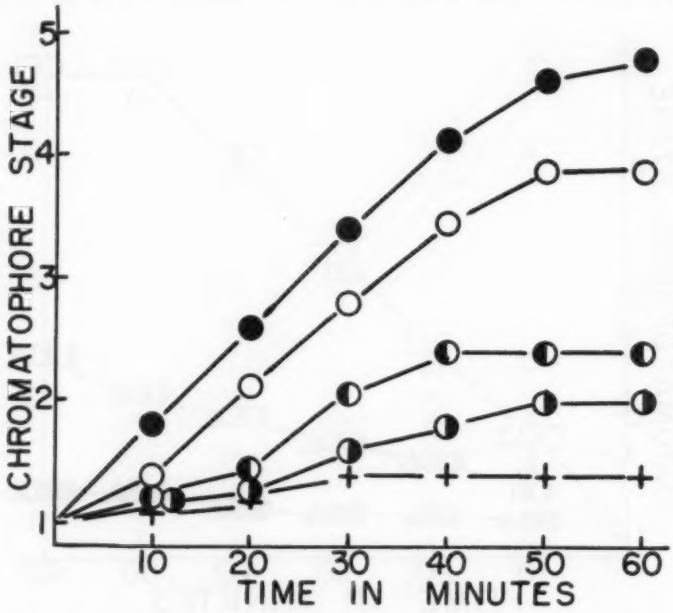

FIGURE 7. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in isotonic sucrose before, +, and after heating at 70° C., ◑, and in boiling water, ◐; and after freezing and thawing, ○. Homogenate in distilled water, ●.

duration of the dispersion of chromatophores in relation to the concentration of the homogenates. The injection of 0.1 ml. of a homogenate of 0.2 sinus gland per ml., *i.e.*, the injection of an amount corresponding to 0.02 sinus gland, is enough to cause a maximal dispersion of the chromatophores in almost 30 minutes and only four hours later have the chromatophores reached the stage of complete concentration again. It is interesting to notice that the time required for normal dark *Uca* to become pale after eyestalk removal is three to four hours. At all concentrations of homogenates dispersion was found to require less time than concentration of pigment within the chromatophores. The elimination of the hormone seems to be a very slow process. Even an injection corresponding to 0.001 sinus gland (open

circles in Fig. 9) induces an effect which disappears completely only after three hours.

In order to obtain some information about the inactivation of the hormone, homogenates of sinus gland in distilled water were incubated with extracts of hepatopancreas, hypodermis and muscle and with one ml. of blood of *Uca*. The extracts were prepared by homogenizing one hepatopancreas, the muscle of one claw, and the hypodermis of the branchiostegites separately, in one ml. of sea water. The blood was removed at the junction of the body and the fourth walking

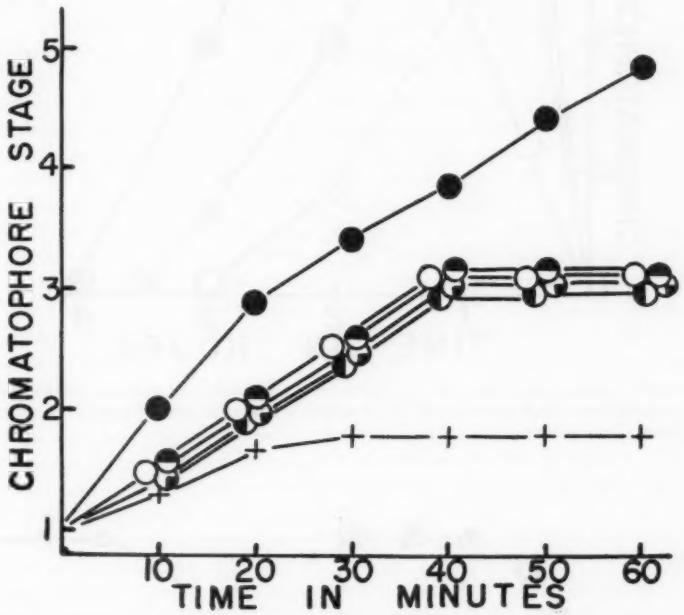

FIGURE 8. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in isotonic sucrose before, +, and after treatment with detergents and digitonin. ◐, Duponol; ◓, saponin; ○, sodium desoxycholate; ◔, digitonin; ●, sodium desoxycholate plus heat and dilution in sea water.

leg, with the aid of a glass pipette. The only extract which caused a complete inactivation of the black chromatophore-dispersing hormone was that of hepatopancreas. After one hour of incubation with extracts of hypodermis or muscle, or with blood, at room temperature, no decrease in the activity of the hormone was observed (Fig. 10a).

The enzyme in the hepatopancreas responsible for its action might be a proteolytic one, since the same effect was obtained when homogenates of sinus glands in distilled water were incubated at 37° C. for one hour with some crystals of chymotrypsin (Fig. 10b). These results suggest that the black chromatophore-dispersing

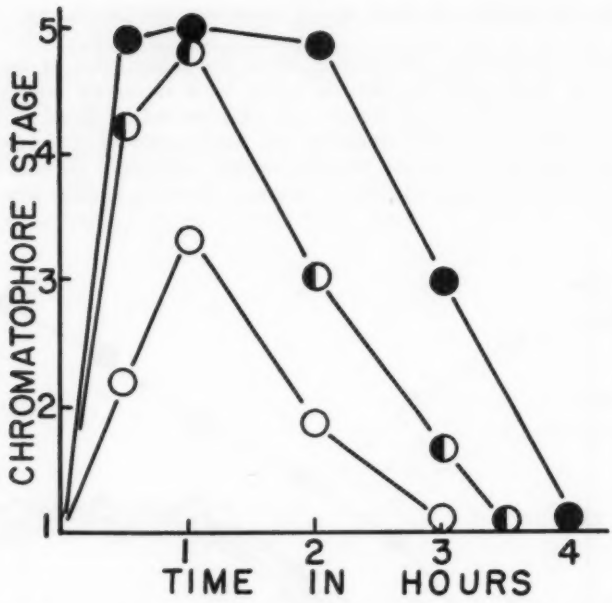

FIGURE 9. Response of black chromatophores of the whole *Uca pugilator* to injections of 0.1 ml. of homogenates of sinus glands in distilled water, in different concentrations: ●, 0.2; ◐, 0.02; ○, 0.001 sinus gland per ml.

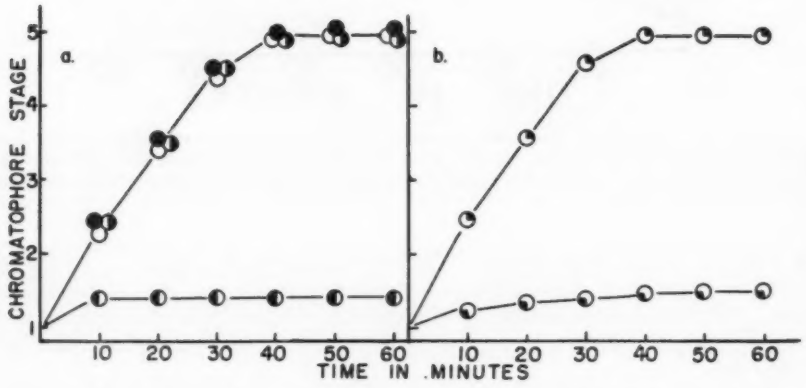

FIGURE 10. Response of black chromatophores of *Uca* to injections of homogenates of sinus glands in distilled water before and after incubation with chymotrypsin for one hour at room temperature and after incubation with chymotrypsin for one hour at 37° C.: ●, muscle; ○, blood; ◑, hypodermis; ◐, hepatopancreas; ◔, distilled water; and ◕, chymotrypsin.

hormone is a polypeptide, but the acceptance of this hypothesis depends upon further experiments.

Discussion

The experiments in section I indicate that in the crab, *Uca pugilator*, the black chromatophore-dispersing hormone is stored in sinus glands within the granules. This assertion is supported by the following observations. First, homogenates of sinus glands in isotonic sucrose have only a small effect on the chromatophores of legs of *Uca pugnax*. These homogenates diluted in distilled water cause a maximal dispersion of the chromatophores, indicating a more or less complete release of the hormone. Second, a sedimentable fraction containing hormone was obtained by centrifugations at the speed of 20,000 × gravity. This centrifugation caused only a partial sedimentation of granules. However the analysis of the activity of homogenates in isotonic sucrose and in distilled water after a series of dilutions shows that the homogenates in sucrose are as effective as those in distilled water ten times more diluted, indicating that only 10% or less of the total amount of hormone is present free in the solution, the other 90% remaining in the granules. Whether this free hormone is already present in sinus glands *in vivo*, or whether it is the effect of the disruption of some granules during the process of homogenizing, is not known. Hillarp, Lagersted and Nilson (1953) have observed that at increased duration of homogenization the catechol content of the granules of the adrenal medulla cells decreases. Berthet and De Duve (1951) have also found that a partial damage to the mitochondria containing acid phosphatase is caused by the process of homogenizing liver tissue. This may be the case with the homogenates of sinus glands.

The effect observed by lowering the tonicity of the medium reinforces the evidence of the presence of the chromatophore-dispersing hormone in granules, and suggests the existence of a semipermeable membrane for the granules. The rapid release of hormone observed when the tonicity of the medium decreases suggests that there is a lysis of the granules, by rapid entrance of water.

The membrane of the granules seems to be freely permeable to ions like sodium and potassium, because the solutions of isotonic sodium chloride, potassium chloride, or sea water cause an immediate and marked release of hormone from the granules. Hillarp and Nilson (1954) have found that the granules of the adrenal medulla can be suspended in sucrose or in certain isotonic electrolyte solutions without a considerable release of catechol amines. Blaschko, Hagen and Welch (1955), however, have observed that in NaCl or KCl an appreciable liberation of adrenaline occurs. Pardoe and Weatherall (1955) also have obtained liberation of vasopressin and oxytocin from granules of the posterior pituitary of rats, by simple dilution in saline of the suspensions of granules in isotonic sucrose. Isotonic saline solutions have been demonstrated to afford only transient osmotic protection for mitochondria of the rat liver (Berthet, Berthet, Appelman and De Duve, 1951; Appelman and De Duve, 1955) and for mitochondria of insect muscle (Watanabe and Williams, 1953). The authors above cited observed also that in media where part of the saline is replaced by isotonic sucrose, the mitochondria are very stable. Similarly, the granules of sinus glands are equally stable in pure sucrose, in a mixture of 25% isotonic sucrose and 75% isotonic salines, and in 2.5% bovine plasma albumin in 0.54 *M* potassium phosphate buffer at pH 7.

Heating, freezing and thawing, and the action of detergents have been proved efficient treatments to release physiologically active substances from granules (Hillarp and Nilson, 1954; Pardoe and Weatherall, 1955). In the case of granules of sinus glands, all these treatments induce a more or less appreciable release of hormone but none of them is sufficient to cause a complete liberation of hormone from the granules.

The hormone in the homogenates in isotonic sucrose after heating, freezing and thawing and after the action of detergents is still present either inside the granules or in such combination that it can not be active. This was proved by the experiments in which parts of the homogenates after these treatments were diluted in sea water, and greater activity was then observed.

These observations may suggest the following hypothesis: that inside the granules the chromatophore-dispersing hormone is found in two forms, bound to a large molecule and as free small molecules. By heating, freezing and thawing and by the action of detergents, the membrane of the granules suffers some disruption, permitting only the passage of the small molecules to the solution. In hypotonic and saline media, which cause a lysis of the granules, all the molecules are present free in the solution. One has to admit also that the hormone is active in both forms, or that once free in the solution, the large molecules disintegrate into the smaller ones. This could explain the different activity of the homogenates of sinus glands in isotonic sucrose after these different treatments.

It is interesting to discuss here the results of Knowles, Carlisle, and Dupont-Raabe (1955) with the chromactivating substances of sinus glands and post-commissure organs of *Leander serratus*, and corpora cardiaca of *Carassius*. By electrophoresis of extracts of these organs they detected the presence of a substance, the "A-substance," which is relatively immobile at pH 7.5 and does not pass through cellophane membranes. This substance concentrates all the red chromatophores of *Leander*. When the extracts are left standing several hours at room temperature, the A-substance disintegrates into others, the a-substances, which have high mobility at pH 7.5 and pass freely through a dialysis membrane. The a-substances affect only the small red chromatophores of *Leander*. They observed also that only the a-substances are released by electrical stimulus of the commissure when the post-commissure organ is in a saline bath.

So, it is reasonable to believe that the dispersing hormone of *Uca* can also be found as large and small molecules and both be active on black chromatophores. But, of course, this is an assumption which depends upon further experiments in this subject.

Heating in boiling water does not cause loss in the activity of the chromatophore-dispersing hormone of *Uca*. Inactivation of the hormone can be achieved, however, by incubation of the homogenates of sinus glands with extracts of hepatopancreas and by the action of the enzyme chymotrypsin. These results suggest that the hormone is a polypeptide.

Carstam (1951) has found that extracts of hepatopancreas of crustaceans and molluscs, and extracts of liver of the guinea pig inactivate the pigment-concentrating hormone of *Leander adspersus*, but he could not obtain the inactivation of the hormone with trypsin. However, Knowles, Carlisle and Dupont-Raabe (1956) have obtained a complete inactivation of the "A-substance" from sinus glands and post-commissure organ of *Leander*, by a crystalline preparation of trypsin and also by a

prolonged acid hydrolysis. Östlund and Fange (1956) have suggested that a chromactivating substance from the eyestalk of *Pandalus* could be an aromatic amine, but in personal communication to Knowles and Carlisle (1956) they have stated that their more recent work indicates that this hormone may possibly be a polypeptide. So far, the studies concerning the nature of the chromactivating substances of crustaceans indicate that they are polypeptides. Porath, Roos, Landgrebe and Mitchell (1955) have isolated a melanophore-stimulating peptide from the pig-pituitary gland. Thus, also in vertebrates the chromatophorotropins seem to be peptides.

Carstam (1951) has also obtained the inactivation of the pigment-concentrating hormone by an enzyme present in the hypodermis of *Leander*. In *Uca pugilator*, in hypodermis as well as in the blood, there was not found an inactivating enzyme for the chromatophore-dispersing hormone.

The experiments where the homogenates of sinus glands in distilled water were injected into the whole *Uca pugilator* show that the response of the black chromatophores is a function of the concentration of the hormone. These results give also an idea about the amount of hormone liberated and its way of action in normal crabs. Insignificant amounts of the hormone (corresponding to 0.02 sinus gland) are enough to induce a maximal dispersion of the chromatophores for a long time. This shows that the elimination or destruction of such small quantities of hormone is a slow process. Stephens, Strickholm and Friedl (1956) have also observed that the dispersing hormone in *Uca* was present in the circulating blood of destalked assay animals in discernible amounts for approximately three hours after injection. Hence, it is reasonable to believe that the dispersing hormone is liberated into the blood in small quantities and eliminated by excretory processes without the interference of special enzymes for its inactivation.

This work has been supported in part by a Rockefeller Foundation fellowship to the author, for a stay in the laboratory of Prof. John H. Welsh, at Harvard University; and in part by Research Grant B-623 from the National Institute of Neurological Diseases and Blindness, National Institutes of Health. I wish to express my sincere appreciation to Prof. John H. Welsh for his skillful assistance during the course of this investigation. I am grateful to Dr. I. Ringler for his help in running the high speed centrifugation experiments.

Summary

1. Homogenates of sinus glands in isotonic sucrose cause little dispersion of black chromatophores when injected into legs or whole *Uca*. A liberation of hormone occurs when homogenates of sinus glands in isotonic sucrose are diluted in distilled water. A fraction, sedimentable by high speed centrifugation, when resuspended in distilled water and injected into the test animals, induces a dispersion of the chromatophores. These results support the view that the black chromatophore-dispersing hormone is contained within granules in sinus glands.

2. The release of the hormone from the granules, obtainable by lowering the tonicity of the medium or by dilution in isotonic saline solutions, suggests that the granules possess a semipermeable membrane.

3. The release of the hormone from the granules is increased by heating, by freezing and thawing, and by the action of detergents and digitonin.

4. The black chromatophore-dispersing hormone may be a polypeptide, since it is inactivated by extracts of hepatopancreas and by chymotrypsin.

5. The rate of disappearance of the hormone from the blood of the crab is very slow.

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THE METABOLISM OF STRONTIUM-90 AND CALCIUM-45 BY LEBISTES

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That fishes accumulate mineral elements from the water in which they swim and incorporate these elements into body tissues has recently been demonstrated by the use of calcium-45 and strontium-89 in fresh water and marine fishes of various species (Prosser *et al.*, 1945; Rosenthal, 1956; Lovelace and Podoliak, 1952; Boroughs *et al.*, 1956; Alexander *et al.*, 1956). The rate of incorporation of calcium-45 into the total body and tissues is linear for *Lebistes* and *Salmo* sp. (Rosenthal, 1956; Lovelace and Podoliak, 1952), but bone and osseous tissues incorporate the nuclide at a greater rate than either visceral organs or muscle. In *Lebistes*, the loss of incorporated calcium-45 from the whole body may be described by at least three separate first-order reactions varying from very fast to very slow, probably reflecting the rate of turnover of visceral organs, muscle, and osseous tissues, respectively. In marine fishes, Boroughs *et al.* (1956) have shown that strontium-89, placed in water, is rapidly incorporated into body tissues and the distribution in tissues is similar to that following oral dosage of the nuclide. These investigators also showed that the rate of excretion of a single oral dose of strontium-89 is rapid during the first few days of the experiment. However, the isotope remaining in the body after the first few days persisted at a constant level for a long time.

In view of the reports that small laboratory mammals (Alexander *et al.*, 1956; Comar *et al.*, 1955), man (Turekian and Kulp, 1956) and marine fishes (Boroughs *et al.*, 1957) discriminate against strontium relative to calcium, and since strontium is chemically similar to calcium, it was of interest to determine the uptake and turnover of strontium-90 by *Lebistes* and to compare this information with that previously obtained with calcium-45. The results of this study form the basis for this report.

Materials and Methods

Adult male wild-type guppies, averaging 125 mg. in weight, were obtained from commercial sources. The experimental design and the treatment of animals and tissues for analysis has been described in detail in a previous publication (Rosenthal, 1956).

All samples for radioactivity assay for strontium-90 were counted after a waiting period of 20 days to permit equilibrium between strontium-90 and its yttrium-90 daughter nuclide. The samples were counted with a windowless gas flow counter to less than a 5 per cent statistical error. Corrections for self-absorption of strontium-90 were made when necessary but the 28-year half-life of this nuclide obviated decay corrections. Assay of calcium-45 was performed as previously described, with the same counting assembly.

No attempt was made to differentiate between strontium-90 and its yttrium-90 daughter, and the use of the term strontium-90 throughout this report refers to the combined activities of strontium-90 and yttrium-90 at equilibrium. The efficiency of the counter for strontium-90 and calcium-45 was determined to be 1.5×10^{10} and 1×10^{9} counts per minute per millicurie, respectively. The strontium-90 and calcium-45 were obtained from the Oak Ridge National Laboratories in the form of carrier-free salts.

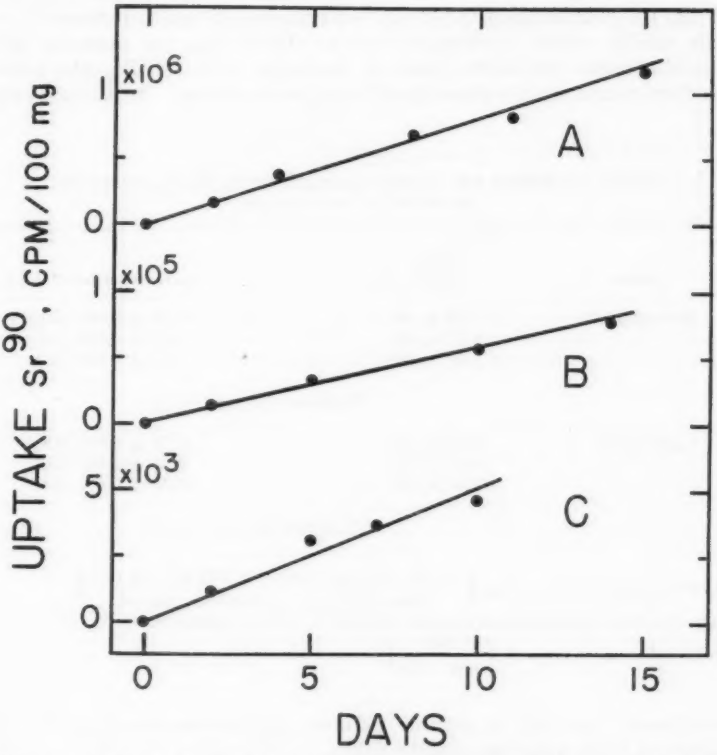

FIGURE 1. Uptake of strontium-90 by male *Lebistes* versus days in water containing the isotope. Each point represents two to four fish. The water activity for Curve A, 1×10^{6} cpm/ml., Curve B, 1.7×10^{5} cpm/ml., Curve C, 1.1×10^{4} cpm/ml.

RESULTS

The rate of uptake of strontium-90 by male *Lebistes* from the water in which they swim was determined by placing the fish in glass aquaria containing 500 milliliters of aged tap water containing the isotope. Distilled water was added daily to compensate for losses of water by evaporation and to maintain the isotope activity of the water within $\pm$ 5 per cent during the experimental period.

The results obtained from these experiments demonstrate the rapid incorporation of strontium-90 into the body of the fish (Fig. 1). The incorporation is linear during a 10- or 15-day experimental period for all concentrations of isotope thus far studied, and the uptake of strontium-90 is similar to the data previously obtained with calcium-45 (Rosenthal, 1956). The similarity between the uptake of strontium-90 and calcium-45 is further shown by calculation of a "concentration factor" which relates the logarithm of the rate of incorporation of the isotope in the body of the fish to the logarithm of the activity of isotope in water (Table I). It is apparent that the concentration factors for both calcium-45 and strontium-90 are surprisingly similar within experimental error. These data are markedly different from those obtained for marine fishes by Boroughs *et al.* (1957), who found that *Tilapia* discriminate against strontium-89 relative to calcium. It is conceivable that

TABLE I

Relationship between rate of uptake and water activity for various nuclides by the body of male Lebistes

Isotope	Water activity cpm/ml.	Concentration factor* ± S.E.
Strontium-90	8.25×10^3	0.70 ± 0.007 (10)**
	1.72×10^5	0.72 ± 0.003 (16)
	1.00×10^6	0.82 ± 0.004 (13)
	Weighted Average	0.75
Calcium-45	8.52×10^3	0.72 ± 0.007 (18)
	9.42×10^4	0.80 ± 0.005 (20)
	7.37×10^5	0.78 ± 0.010 (13)
	Weighted Average	0.77

* Concentration Factor $= \log\left(\frac{\text{Uptake in count/min. per 100 mg. per day}}{\text{Water activity in count/min. per ml.}}\right)$.

** The numbers in parentheses indicate number of animals analyzed.

S.E. = Standard error $= \left(\frac{\Sigma d^2}{n(n-1)}\right)^{\frac{1}{2}}$.

these differences are due to entirely different mechanisms involving the osmotic physiology of fresh water and marine fishes.

The various organs of the body such as the spine, head, viscera, and muscle also take up strontium-90 in a linear fashion during a 10-day experimental period, but the rate of uptake differs for each organ, as shown in Figure 2. Highly mineralized tissues such as the spine and head accumulate strontium-90 at a greater rate than the total body while soft tissues (muscle and viscera) accumulate less of the isotope. The accumulation of strontium-90 by the tissues of *Lebistes* is qualitatively similar to that of calcium. Although the total body accumulates the same amount of calcium-45 and strontium-90, the ratio of organ isotope concentration to total body isotope concentration for strontium-90 differs significantly from that of calcium-45 in all of the organs studied (Table II). Thus it is apparent that the spine, head

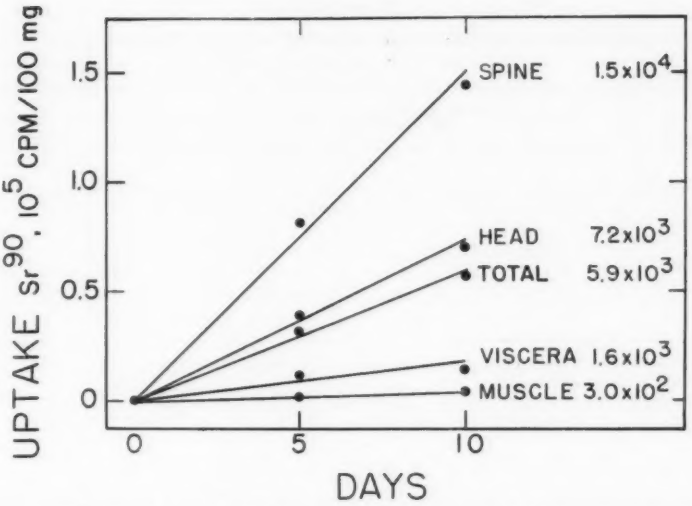

FIGURE 2. Uptake of strontium-90 by various tissues of male *Lebistes* versus days in water containing 9×10^4 counts per minute per milliliter. Values for each tissue represent rate of uptake of strontium-90 in terms of counts per minute per 100 milligrams per day. Each point represents 6 values.

and viscera accumulate significantly more strontium-90 than calcium-45 on a concentration basis. Muscle tissue, on the other hand, tends to incorporate somewhat less strontium-90 than calcium-45. A comparison of the total distribution of strontium-90 and calcium-45 in the various tissues of the body following 10 days of uptake of the isotope from water (Table III) is consistent with the data based on

TABLE II

Relative uptake of strontium-90 and calcium-45 by tissues of male Lebistes

Tissue	Strontium-90*	Calcium-45*	"t"	"P"
Carcass	1.00 ± 0.026** (16)***	1.00 ± 0.045** (20)	—	—
Head	1.28 ± 0.051 (18)	1.07 ± 0.039 (19)	3.29	<0.01
Viscera	0.96 ± 0.118 (18)	0.59 ± 0.087 (21)	2.49	<0.02
Muscle	0.061 ± 0.020 (17)	0.102 ± 0.024 (20)	3.95	<0.01
Spine	2.28 ± 0.10 (15)	1.87 ± 0.10 (16)	3.62	<0.01

* The values represent the ratio $\frac{\text{cpm/100 mg. tissue}}{\text{cpm/100 mg. carcass}}$ derived from 3 to 5 experiments in which the water activity varied from 10^4 to 10^6 cpm/ml. for each isotope.

** Standard error.

*** The numbers in parentheses indicate number of fish analyzed.

$$\text{Students "t" value} = \left[\frac{M_1 - M_2}{\left(\frac{\Sigma d_1^2 + \Sigma d_2^2}{N_1 + N_2 - 2}\right)^{\frac{1}{2}}}\right]\left[\left(\frac{N_1 N_2}{N_1 + N_2}\right)^{\frac{1}{2}}\right].$$

TABLE III

Distribution of strontium-90 and calcium-45 in tissues of male Lebistes after 10 days in isotopic water

Tissue	Isotope distribution ± S.E. (per cent of total)		"t"	"P"
	Strontium-90	Calcium-45†		
Carcass	100.0 ± 10.11 (13)*	100.0 ± 2.92 (15)*	—	—
Head	29.9 ± 0.97 (13)	21.3 ± 1.12 (14)	5.71	<0.01
Viscera	13.5 ± 1.87 (13)	7.3 ± 0.48 (14)	3.34	<0.01
Muscle***	2.8 ± 0.29 (12)	3.7 ± 0.31 (14)	2.12	<0.05
Spine	6.9 ± 0.61 (12)	6.2 ± 0.36 (14)	1.02	—
Remainder**	46.9 ± 2.24 (12)	61.5 ± 2.32 (13)	4.36	<0.01

* The numbers in parentheses indicate number of animals analyzed.
** Calculated by difference.
*** Muscle tissues estimated to comprise 40 per cent of body weight.
† From Rosenthal (1956).

concentration shown in Table II. The apparent discrepancy for the similarity between the distribution of calcium-45 and strontium-90 in the spine (Table III) and the relative uptake of the two isotopes by the spine on a concentration basis is due, in all probability, to our inability to always remove the entire spine from these small fishes. This unavoidable error introduces some uncertainty into the distribution data for the spines.

The head and "remainder" (skin, scales and fins) account for 77 per cent of the total body strontium-90, while these tissues account for almost 83 per cent of the total body calcium-45. Muscle contains the smallest proportion of the total body strontium and calcium (2.8 and 3.7 per cent, respectively) while occupying about 40 per cent of the total body weight. The spine, representing less than 3 per cent of the body weight, contains between 6 and 7 per cent of the total calcium and strontium nuclides, respectively. This comparison between the distribution of strontium-90 and calcium-45, under the same experimental conditions, indicates that the head and viscera incorporate, respectively, 25 per cent and 46 per cent more strontium-90 than calcium-45, while muscle and the remaining tissues accu-

TABLE IV

Relative proportions of tissues of male Lebistes

Tissue	No. of determinations	Per cent of body weight
Head	39	20.6 ± 0.22*
Viscera	38	12.3 ± 0.37
Spine	21	2.8 ± 0.07
Remainder**	39	24.4 ± 0.25
Muscle	—	40.0 (estimated)

* Standard error.
** Remainder includes skin, fins, scales and is calculated by difference assuming that muscle comprises 40 per cent of body weight.

mulate about 25 per cent less strontium-90 than calcium-45. These differences in tissue uptake of the two nuclides are statistically significant and further accentuate subtle differences in the metabolism of these two elements.

Since the relationship of the weight of organs to body weight has not, to my knowledge, been previously determined or published, for *Lebistes*, the relative proportions of the various tissues analyzed in this study are shown in Table IV.

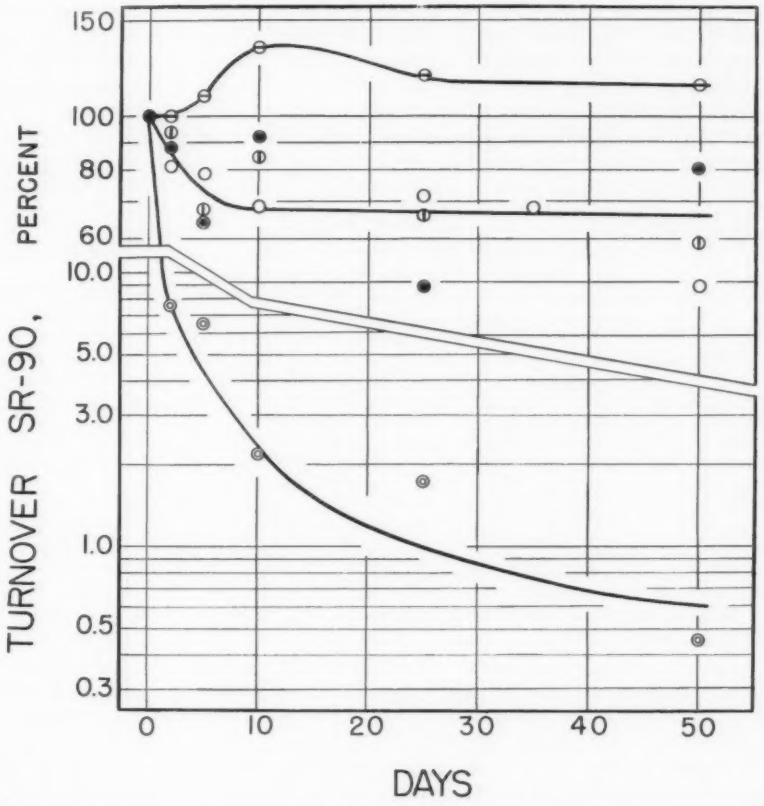

FIGURE 3. Turnover of strontium-90 by various tissues of male *Lebistes* versus days in water containing no isotope. Each point represents 4 to 11 values obtained from 3 experiments. ⊖, spine: ○, body: ●, muscle: ⦶, head: ⊙, viscera. The fish contained about 10^4 cpm/100 mg. on day zero of turnover.

The rate of turnover of strontium-90 by the body and tissues of *Lebistes* was determined by first placing the animals in isotope-containing water for 10 days in order to incorporate sufficient radioactivity into the tissues. After this period the fishes were transferred to isotope-free water which was changed periodically and they were sacrificed at suitable intervals previously described in detail (Rosenthal,

1956). During a 50-day experimental period, the loss of strontium-90 from the total fish could be resolved into two components that may be described by first order reactions (Fig. 3). The first component, turning over rapidly with a biological half-life of about 8 days, represents loosely-bound strontium-90 in visceral tissues of the body. This is somewhat longer than the three-day half-life for the rate of turnover of the fast component with calcium-45 (Rosenthal, 1956). The loss of strontium-90 by the viscera is extremely rapid, so that 92 per cent of the radioactivity is lost during the first two days of the experiment. The second component has an exceedingly long half-life of about two years or more. During a similar experimental period with calcium-45 (Rosenthal, 1956) three components with biological half-lives of 3 days, 137 days, and 309 days were apparent which reflect the turnover rates of visceral tissues, muscle and carcass, respectively. The absence of an intermediate component for strontium-90 is primarily due to the very slow turnover rate of strontium-90 by muscle tissue, and to a lesser extent, the head.

TABLE V

Distribution of strontium-90 and calcium-45 in tissues of male Lebistes after 40–50 days of turnover in non-isotopic water

Tissue	Isotope distribution ± S.E. (per cent of total)		"t"	"P"
	Strontium-90 (50 days)	Calcium-45 (40 days)†		
Carcass	100.0 ± 11.2 (5)*	100.0 ± 5.48 (8)*	—	—
Head	42.7 ± 5.16 (5)	34.4 ± 2.08 (7)	1.69	>0.15
Viscera	0.2 ± 0.06 (5)	0.5 ± 0.04 (5)	2.12	>0.05
Muscle***	3.0 ± 0.87 (5)	2.6 ± 0.46 (5)	0.56	NS
Spine	21.0 ± 4.47 (5)	19.4 ± 1.04 (7)	0.41	NS
Remainder**	33.1 ± 7.79 (5)	43.1 ± 2.38 (6)	1.33	>0.20

* The numbers in parentheses indicate number of animals analyzed.
** Calculated by difference.
*** Muscle tissue estimated to comprise 40 per cent of body weight.
† From Rosenthal (1956).

It is of interest to note that muscle tissue strontium-90 with a biological half-life of about two years as calculated from the last 25 days of the experiment, is lost in a manner similar to that of the total body. This is in contrast to the biological half-life for muscle of 137 days as determined previously for calcium-45, and it would appear that the metabolism of the two elements differs in muscle tissue. The exceptionally slow turnover of strontium-90 in muscle tissue of marine fishes has recently been observed by Boroughs *et al.* (1956).

The spine, which consists not only of mineral matter but also of intervertebral cartilage, tendon, and organic bone matrix, continues to incorporate the isotope for about 10 days after the fish is placed in isotope-free water. The additional nuclide must be derived from a redistribution of isotope from soft tissues such as viscera. Similar data were obtained with calcium-45 (Rosenthal, 1956). The accumulated isotope does not remain fixed in the spine, however, but is subsequently lost and a new equilibrium consistent with that of the mineral component of bone becomes

established. The biological half-life of strontium-90 in the spine, calculated during the last 25 days of the experiment, may be estimated to exceed two years, a value consistent with the biological half-life of calcium-45 previously determined (Rosenthal, 1956). The additional increase of the strontium-90 and calcium-45 in the bone and its relatively rapid loss may represent a rather labile binding-site ($T\frac{1}{2}$ = about 50 days) for bone formation.

The distribution of strontium-90 in various organs and tissues of the body after 50 days in isotope-free water is compared with the distribution of calcium-45 after 40 days in isotope-free water (Table V). It is interesting to note that the distributions of both isotopes at the end of 40 days of turnover for calcium-45 and 50 days for strontium-90 are not significantly different. A comparison of the rate of turnover of strontium-90 by the tissues, shown in Figure 3 of this report, with the rate of turnover of calcium-45 previously published (Rosenthal, 1956) indicates that the similarity of distribution of both isotopes at these particular time intervals is coincidental. Extrapolation of the turnover rates for both isotopes indicates that the head, muscle and spine would retain a greater proportion of the body strontium-90, while the viscera and "remainder" would contain less strontium-90 throughout the life of the fish.

Discussion

It is apparent from these studies that fresh water fishes accumulate strontium-90 from the water in which they swim and that the rate of uptake is similar to that of calcium-45. Moreover, we have recently shown that the rate of uptake of strontium-90 and calcium-45 by other fresh water fishes (*Danio* and *Tanichthys*) is similar to the data we have obtained with *Lebistes*. These data differ from the studies of Boroughs *et al.* (1957), who found that marine fishes discriminate against strontium-89. This apparent disagreement may be due to marked differences in osmotic regulation between marine and fresh water fishes. On the other hand, discrimination of strontium isotopes relative to calcium by small laboratory mammals appears to be well documented (Comar *et al.*, 1955; Turekian and Kulp, 1956; Comar *et al.*, 1956). Comar *et al.*, (1956) have indicated that the processes of major discrimination, in rats, are decreased absorption of strontium from the intestinal tract and increased urinary strontium excretion, processes which cannot be measured directly in small fishes. These two processes would tend to limit the quantity of strontium entering the body and its retention in the body, but in fishes, the gills play a major role for the absorption and excretion of mineral elements. The similarity of the "concentration factor" (Table I) for both nuclides by the total body of the fish indicates no discrimination of strontium-90 by these fishes. It is possible that subtle differences between strontium-90 and calcium-45 uptake by fishes may become apparent by the use of differential methods in which both nuclides are present in the same medium. These and other aspects of the problem are under investigation.

The rate of excretion of strontium-90 by the total body and tissues (except viscera) of *Lebistes* is slower than that of calcium-45. The data appear to be in contrast with the report by Lengeman (1957), who showed that rat bones, *in vitro*, lose more strontium-90 than calcium-45. Since the accumulation and retention of mineral elements into bone depend on the rate of bone formation and sequestration of elements into slowly exchanging bone matrix, comparisons between such diverse biological systems may be hazardous. Nonetheless, tissues of *Lebistes* rich in cal-

cium, such as the spine and head, accumulate and retain a larger percentage of the total body strontium-90 than that of calcium-45, in accord with the studies of Lengeman (1957) and Comar *et al.* (1956) for rats. Although no explanation is offered at this time concerning the mechanism of incorporation of alkaline earth elements from water by fishes, the similarity of uptake of calcium-45 and strontium-90 by fresh water fishes (Rosenthal, 1957) indicates a fundamental and essentially similar process.

I am grateful to Mari Lou Pfluke, Helen Cundiff and Paul Myers for their technical assistance and to E. Pfluke, Pfluke's Aquarium and Pet Shop, Rochester, N. Y. for animals and supplies. This study was aided by a grant, contract No. AT (30-1)-1712, from the Atomic Energy Commission.

Summary

1. The uptake of strontium-90 by male *Lebistes* from the water in which they swim is linear with time for the total carcass and tissues studied. Tissues containing high concentrations of calcium accumulate more strontium-90 than soft tissues. The rate of turnover of the nuclide varies from very fast to very slow according to the type of tissue. The whole body, head and spine retain strontium-90 for long periods of time ($T\frac{1}{2} = 600$ days) while viscera loses the isotope rapidly ($T\frac{1}{2} = 8$ days).

2. A comparison between strontium-90 and calcium-45 uptake and turnover by male *Lebistes* are qualitatively similar but significant quantitative differences are apparent.

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THE MOLTING CYCLE OF THE SPINY LOBSTER, PANULIRUS ARGUS LATREILLE. IV. POST-ECDYSIAL HISTOLOGICAL AND HISTOCHEMICAL CHANGES IN THE HEPATO-PANCREAS AND INTEGUMENTAL TISSUES [1]

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Following molt, the major tasks which confront the crustacean are growth of the soft tissues and the continued accretionary growth and hardening of the skeleton by deposition of mineral salts therein. In spiny lobsters of 80–89 mm. carapace length, weight stability is not achieved until 28–35 days following molt (late Stage C) (Travis, 1954). This is a period at which the skeleton is fully hardened, water content is normal, and presumably growth of the tissues is fairly stable. During the early postmolt period, however, when rapid accretionary growth and calcification of the skeleton are occurring, marked changes are observed in the hepatopancreas and integumental tissues. Accordingly, the present paper will be concerned with those marked changes in the hepatopancreas and integumental tissues which occur concomitantly with the development and calcification of the post-exuvial layers of the skeleton.

Materials and Methods

Animals

Male and female spiny lobsters ranging in carapace length from 80–89 mm. were obtained and handled as previously described (Travis, 1954).

Designation of stages in the molting cycle

Stages of the molting cycle were designated by time intervals, in days, as previously described (Travis, 1955a) and by the method of Drach (1939). For *Panulirus argus,* Stage A through C encompasses a period of approximately 51 days during the summer months. Stage A, immediately following molt and the stage in which the principal layer begins to be deposited, has a duration of approximately 24 hours or one day. Stage B, beginning calcification, continued thickening of the principal layer and preliminary hardening of the skeleton, is approximately six days

[1] This work was supported in part by an E. L. Mark Fellowship from Harvard University, a Mees Fellowship from the Bermuda Biological Station, and an Atomic Energy Commission Pre- and Postdoctoral Fellowship. The author wishes to express her sincere appreciation to Dr. John H. Welsh, Dr. E. E. Mortensen, and Dr. A. B. Dawson for their most helpful criticism and suggestions regarding this manuscript.

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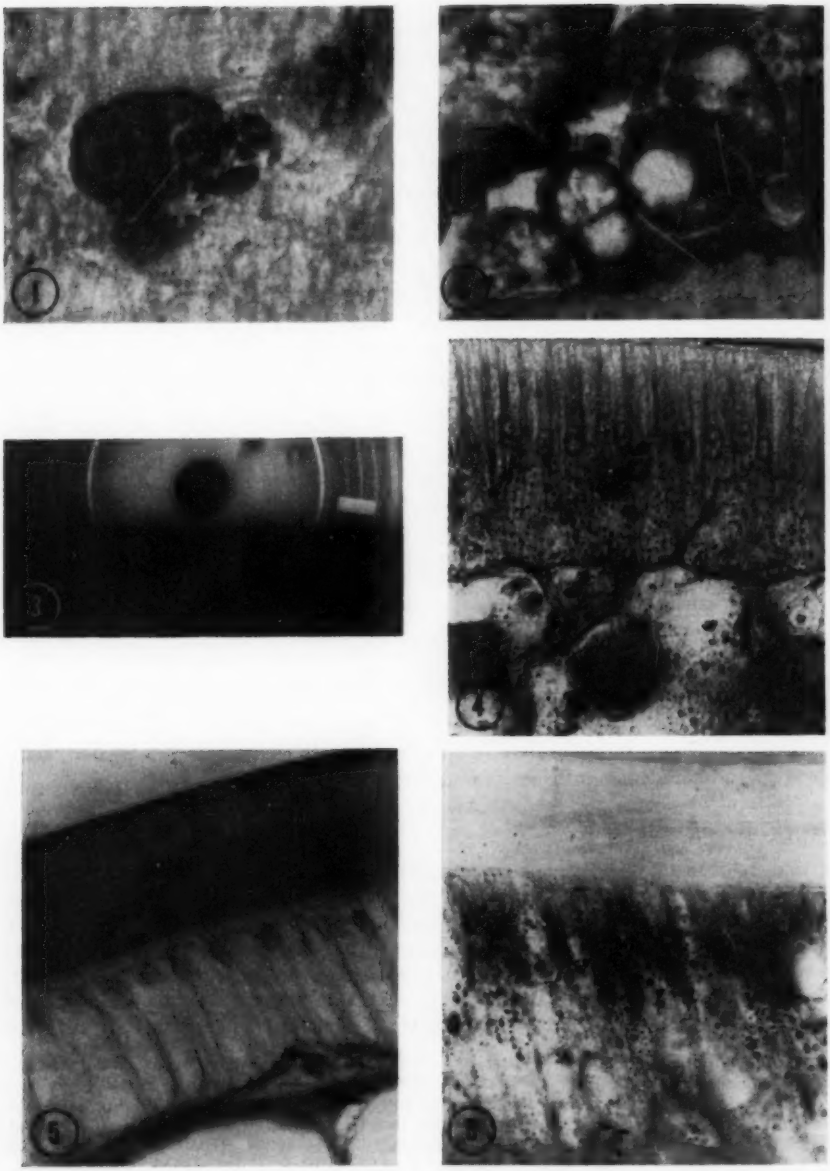


FIGS. 1–6.

in length, existing from two through seven days following molt. Stage C, a stage in which the principal layer and the new membranous layer are completed and in which the skeleton is completely hardened, is the longest period of the molting cycle (44 days), existing from approximately the eighth day through the fifty-first day following molt.

Histological and histochemical methods

For the histological and histochemical studies, pieces of integument and integumental tissues were removed from the carapace of *Panulirus* (see Figure 1; Travis, 1955a) on each of eight consecutive days following molt. Likewise the right posterior lobe of the hepatopancreas was removed on each of seven consecutive days following molt. Tissues from three animals were used to represent each of these days with the exception of the first, fourth, and eighth day following molt for the integumental tissues. In these cases, tissues from one animal were used.

Most integumental tissues were embedded in celloidin and cut at 10 μ. The posterior lobe of the hepatopancreas was embedded in paraffin and cut at 8 μ, with the exception of hepatopancreatic tissues fixed and embedded for lipid detection.

Portions of the integument and hepatopancreas fixed in Helly's and alcoholic Bouin's fluid were stained by the following methods:

1. Mallory's triple stain
2. Periodic acid Schiff (PAS) of McManus, as described by Lillie (1948)
3. Bensley and Bensley's method (1938), for demonstrating muco- or glycoprotein by means of toluidine blue (see Travis, 1955a).

For detection of calcium deposits, portions of the skeleton were fixed in nine parts of 95% alcohol and one part of 40% formaldehyde, and were stained with the following:

1. Mallory's triple stain
2. Schmorl's purpurin (Lillie, 1948)

FIGURE 1. A reserve cell, "mulberry"-like in appearance, of the sub-epidermal connective tissue from an animal three days following molt. Note lumpy or stainable balls of material (arrow) of mucopolysaccharide as well as calcium. 800 ×.

FIGURE 2. Large oval reserve cells of the hepatopancreas showing the presence of large vacuoles (arrows), some of which contain flaky or granular-like stainable material while others appear clear, a condition observed from five through seven days following molt and possibly correlated with a marked decrease in mucopolysaccharide and calcium. 800 ×.

FIGURE 3. X-ray diffraction photograph taken of dry powder obtained from triturated pieces of the area of softening. The presence of calcite lines reveals that calcium carbonate is present in the spiny lobster skeleton as calcite, not aragonite or amorphous calcium carbonate.

FIGURE 4. Glycogen distribution in the epidermis of the outer integument and sub-epidermal connective tissue at one day postmolt. Note small number of glycogen granules localized in the proximal half of the outer epidermal cells. At this same time abundant amounts are concentrated at the bases of the epidermal cells of the inner integument. 760 ×.

FIGURE 5. Note that at two days postmolt glycogen has completely disappeared from the epidermis of the inner integument and is not observed again in this tissue during the entire postmolt period. 760 ×.

FIGURE 6. The heavy concentration of glycogen observed in the distal half of the outer epidermal cells during a period of two through four days following molt. Little glycogen at this time is present in the sub-epidermal connective tissue. 760 ×.

3. Alizarin red S (Manigault, 1939)
4. Von Kossa's method (Lillie, 1948). Before following this procedure, tissues were washed in 5% aqueous KNO_3 for five minutes or more to remove the chloride present. With Von Kossa's method, the silver from the silver nitrate is precipitated as phosphate on the surface of calcium phosphate granules. The silver phosphate is reduced in the presence of light to metallic silver, forming a black crust on the surface of calcium phosphate granules. Mallory (1942) states that calcium carbonate granules become coated with silver carbonate, which in sunlight gives off CO_2 and leaves a black silver oxide on the surface of these granules. These reactions may not occur in the presence of organic substances (Lison, 1953) nor in the presence of quantities of chloride (Lee, 1946).

   Parts of the epicuticle, pigmented layer and principal layer appear either black or brown with Von Kossa's method. Cameron (1930) pointed out that the most recent calcium deposits in teeth appear dark brown by this method, while the older calcified layers appear lighter brown. Some calcified areas, he noted, did not stain at all. It should be pointed out that this method has the great advantage over alizarin and purpurin in that it enables one to visualize calcium deposits in granular form. It is, therefore, excellent for the detection of skeletal deposits whereas the latter methods are not.
5. Microincineration (Scott, 1933) was used to confirm the presence of calcium deposits detected by the stains mentioned above. The white calcium ash under dark-field illimination appeared in the same areas as indicated by the stains. To confirm this as being calcium ash, the gypsum test was used.

   For detection of calcium in the hepatopancreas, tissues were fixed in the same fashion as the integumental tissues and were stained with alizarin red S.

For detection of alkaline phosphatase, integumental and hepatopancreatic tissues were fixed in cold 80% alcohol, and embedded in paraffin. Alkaline phosphatase was determined by the method of Gomori (1941). Control sections were made using the incubating medium without added substrate. This method is extremely useful for the detection of calcium deposits. In control sections which do not show the presence of alkaline phosphatase, calcium deposits, if present, show up remarkably well.

Only the hepatopancreas was used for the detection of lipids. Portions of the tissue, in this case, were fixed in 10% neutral formalin and were imbedded in carbowax (method of Blank and McCarthy, 1950), cut at 10 and 15 μ and stained for lipids with Sudan black B.

Observations

The Postmolt Animal (Stage A and B)

1. *The integument and integumental tissues*

   a. *Tissues*

During the early post-ecdysial period (Stage A and B) pieces of exoskeleton with attached integumental tissues were removed from the lateral portion of the carapace (see Fig. 1; Travis, 1955a). The lateral portions of the carapace of

Crustacea, as one will recall, are folded in such a way that there is an outer epidermis and integument, (the outer integument being in contact with the surrounding sea water) as well as an inner epidermis and integument (the inner integument facing the gills, in contact with the sea water in the branchial chamber). Sections of the exoskeleton with attached integumental tissues indicate that the epidermal cells of the outer integument remain extremely long and attenuated and indeed fibrillar in nature. This condition is similar to that observed in the late premolt animal. The markedly fibrillar nature of the outer epidermis, however, is apparent in all stages of the molting cycle. Nuclei in these epidermal cells of the outer integument are central (Fig. 4) whereas those of the inner integument are more distal in location (Fig. 5). The inner epidermal cells, also somewhat fibrillar in nature, remain about half the length of the outer epidermal cells during all stages of the molting cycle (see figures from Travis, 1955a).

Both the outer and inner epidermis, during the early postmolt period (Stage A and B), show a gamma metachromasia (pink-purple) with toluidine blue, indicating the presence of a glyco- or mucoprotein. The presence of phosphatase, glycogen, and calcium in these tissues during the early post-molt period will be discussed in a subsequent section of this paper.

As was pointed out (Travis, 1955a), the sub-epidermal connective tissue is of a loose spongy type.

The large oval reserve cells, described as "protein cells" by Cuénot (1893) and resembling Leydig Cells, Type I (Kükenthal, 1926–1927), constitute by far the most prominent and most interesting cell types within this sub-epidermal connective tissue. These reserve cells vary greatly in structural appearance during the molting cycle. When storing reserves they become greatly swollen and may take on a "mulberry" appearance (Fig. 1). When devoid of reserves they may decrease in size with their vacuoles becoming clear or containing flaky or granular-like stainable material. Since the reserve cells are found within the tissue spaces among other Leydig cells, they should not, perhaps, be considered as permanent structures within this tissue. This has become somewhat clearer from the work of Sewell (1955), in which he points out that the origin of these reserve cells, which he calls "lipo-protein cells," in *Carcinus* is from amoebocytes and that possibly they revert to amoebocytes following molt. This suggestion could account for their cyclic peaks and declines in size and abundance, and changes in structural appearance at daily intervals during the early postmolt period of *Panulirus*, as indicated below.

For the sake of comparison, the reserve cells in *intermolt animals* (*late Stage C*) constitute the most prominent cell-types within the connective tissue. They are large oval cells, vesicular in nature, with a capsule-like envelope of cytoplasm and a peripheral nucleus (see figures; Travis, 1955a). They range in size from 24–51 μ with an average size of 32 μ. After alcoholic Bouin's fixation they stain blue-gray with Mallory's triple stain, the vacuoles in this case containing blue-gray flaky or granular-like material; with the PAS method the entire cell is a deep pink-purple color; with toluidine blue these cells stain either blue-gray or green-gray. After A–F (alcoholic formaldehyde) fixation they similarly stain blue-gray with Mallory's triple stain, but do not stain with alizarin red S, purpurin or the Von Kossa method. With these three latter stains the reserve cells could be easily overlooked.

Similarly, for the sake of comparison reserve cells in *premolt animals* (*Stage D*) range in size from 30–51 μ with an average size of around 40 μ. Structurally, they maintain their oval appearance but stainable material within the cells is lumpy and might well be described as consisting of rather discrete spheres (Fig. 1). After alcoholic Bouin's fixation, the cells again stain blue-gray with Mallory's but do not show clearly the speres of stainable material; with PAS they again stain deep pink-purple and show clearly the discrete balls of material; with toluidine blue the balls of material are yellow-green in color and refractile in nature. Following A–F fixation, the cells again fail to stain with alizarin red S, purpurin and the Von Kossa method.

From one through seven days following molt (Stage A and B), the reserve cells appear to undergo cyclic peaks and declines in size and abundance, and the storing of reserves.

At *one day* following molt (Stage A) the reserve cells remain approximately the same size as those observed in the premolt animal, a range in size from 32–48 μ and an average size of 36 μ. However, there would appear to be a slight decrease in number. Following alcoholic Bouin's fixation, they stain in much the same fashion as that observed in the premolt animal, although the spheres of stainable material are not as apparent. They fail to stain, following A–F fixation, with the same stains mentioned in the premolt animal.

On the *second day* following molt (beginning of Stage B) the reserve cells have greatly decreased in number and size. They range in size from 12–29 μ with an average of around 19 μ. When observed, their vacuoles are clear and vesicular, lacking the lumpy balls of material, with the exception of very small spheres at their periphery. They stain in a similar manner to those observed on the first day with the exception of the fact that a few show a very small number of calcium granules after the Von Kossa method.

On the *third day* the reserve cells again are present in great numbers, comparable to the condition observed in the premolt animals. They range in size from 32–45 μ with an average size of around 38 μ, which compares favorably with the average size observed in the pre- or intermolt animal. The reserve cells at this time take on an irregular "mulberry" appearance by enclosing large stainable spheres of material within their vacuoles (Fig. 1). Further, their staining properties change markedly. Following Bouin's fixation, they again stain blue-gray with Mallory's, deep pink-purple with PAS and yellow-green with toluidine blue. Following A–F fixation, they stain for the first time, a brilliant orange-red with Mallory's scarlet with alizarin red S and purpurin, and yellow-brown with Von Kossa's method. This indicates that not only is muco-polysaccharide, possibly muco- or glycoprotein, bound by these cells at this time but that they are filled with calcium, which is distinctly apparent with stains used for this purpose.

Interestingly enough, by *the fourth day* the reserve cells are hardly apparent. The large stainable balls of material are lacking and the few cells present are smaller in size, ranging from 19–29 μ with an average size of about 26 μ. In the few apparent cells, there is little evidence of either mucopolysaccharide material or calcium.

On the *fifth day*, these cells again reach a peak in abundance and size. With all stains and fixatives used, they are similar in every way to the three-day condition, with the exception of the fact that the mucopolysaccharide present stains only faintly with PAS, possibly indicating a decrease in concentration of the muco-

polysaccharide material or the unavailability of its reactive groups to PAS. The cells range in size from 29–42 μ with an average of around 34 μ, are "mulberry-like" in appearance, and are again filled with calcium which shows up after appropriate fixation and staining.

By *six days*, the cells are fewer in number, somewhat smaller (range 17–27 μ, average 23 μ), but similar in staining properties to the fifth-day condition.

On the *seventh day* (end of Stage B) the cells are few in number but are somewhat larger in size (35–40 μ, average about 37 μ), and are detected after Bouin's fixation and toluidine blue staining and following A–F fixation and Mallory's, alizarin red S, purpurin, and Von Kossa's method, indicating again that these cells are loaded with calcium.

By *eight days* following molt (beginning of Stage C), the reserve cells are again not apparent.

It is, therefore, evident that the reserve cells even within the early postmolt period, Stage A and B, undergo, at daily intervals, cyclic peaks and declines in size and abundance, changes in structural appearance, and staining properties. The polysaccharide material which is distinctly evident up to the fifth day following molt is always diastase-fast and colors deep pink-purple with PAS but does not show gamma metachromasia with toluidine blue. Pearse (1953) has suggested that polysaccharide material staining in this way with PAS and frequently failing to show gamma metachromasia with toluidine blue probably indicates the presence of either a muco- or glycoprotein. As has been mentioned previously (Travis, 1955a) the reserve cells of late Stage C and Stage D animals stain with PAS in the same manner. This polysaccharide material probably represents reserve substances for the new skeleton and may, as was pointed out by Travis (1955a), during the premolt period represent breakdown products from the old skeleton.

None of the integumental tissues of *Panulirus*, unfortunately, were fixed for the detection of lipids. As was pointed out by Travis (1955a) the reserve cells within the connective tissue of the hepatopancreas contain much lipid and it would likewise be expected that the reserve cells of the sub-epidermal tissue also store it. Sewell (1955) has definitely shown that these reserve cells beneath the connective tissue of *Carcinus* do indeed store lipoprotein. These lipoprotein reserves reach a maximum in C_4 (late Stage C) and early D (D_1) and then begin to decrease, apparently as lipid content of the epidermis increases. These cells, as Sewell suggests, begin to disappear after the pre-exuvial layers of the skeleton are deposited and completely disappear by the end of Stage B. However, reserve cells of *Panulirus* become filled with calcium on the third, fifth, sixth, and seventh day following molt whereas on the first, second, and eighth day either no calcium or very little was apparent in the reserve cells. This would suggest to the present author cyclic peaks in calcium storage alternating with cyclic release to the epidermis as calcification of the skeleton occurs. The present author would expect this cyclic process (peaks and declines in size and abundance, changes in structural appearance, and calcium binding and release) to continue throughout early and middle Stage C, since calcification of the skeleton is not fully completed for at least three weeks following molt. If, therefore, the reserve cells arise from amoebocytes, as Sewell (1955) suggests, and possibly revert to amoebocytes after they have discharged their reserves, such a situation could clearly account for the cyclic peaks in size and abundance at varying daily intervals within a single stage of the molting cycle.

In this sense, the present author would be inclined to accept Sewell's suggestion that the reserve cells represent phases of activity of the amoebocyts with peaks not only before molting, as Sewell suggests, but following molt as well. The reserve cell cycle would then correspond more closely with the oenocyte cycle, being present throughout the molting cycle but reaching peaks at various phases of it. It might be pointed out that days in which reserve cells are scarcely apparent (2, 4, and 8 days following molt), large numbers of amoebocytes are apparent in the sub-epidermal connective tissue.

b. *The integument*

During the early postmolt period (Stage A and B) the post-exuvial layers of the skeleton are deposited. Of the post-exuvial layers of the outer integument, only the principal layer or calcified zone is progressively thickened during Stages A and B.

The amount of skeletal material, in total thickness, deposited per day, in the area of carapace from which sections of the integument were cut, during Stages A and B, varies from 14–72 μ with an average of around 38 μ. The thickness of the principal layer when fully formed varies from around 460–550 μ in animals used in this investigation (80–89 mm. carapace length). If one assumes that a constant amount of skeletal material is deposited daily in the principal layer, for example 38 μ, one can, by using this figure and the total thickness of the fully formed principal layer, roughly calculate the time in days when this layer is completed. By using these values, the calculated time at which the principal layer is completed would be around 15 days following molt. This time, however, is in actuality closer to 20 days following molt. Therefore, the membranous or non-calcified layer would not apparently begin to be deposited before the third week following molt (Stage C_3 of Drach, 1939). By the fourth week following molt (late Stage C or C_4 of Drach) the membranous layer is fully formed (Travis, 1955a).

The pigmented layer (toluidine blue-staining) shows the presence of a muco- or glycoprotein one through five days following molt but by the end of this period the tinctorial properties of this layer have decidedly changed. A deep purple rather than a pink-purple is given with this stain, indicating that the properties of the protein and closely associated chitin units of the pigmented zone have been changed by quinones. The newly formed principal layers initially show a green coloration with toluidine blue but shortly take on a pink coloration. The membranous layer, when fully formed, is light green in color with toluidine blue, possibly indicating differences in the organic nature or composition of this and the principal layers.

The inner integument, bordering the gill chamber, undergoes little if any thickening after the second day following molt. It attains a total thickness of approximately 30 μ, that of a late Stage C animal, by the second day following molt, indicating that the inner integument is completed during a period of three days preceding molt and two days following molt (Travis, 1955a).

As the post-exuvial layers are deposited during the early postmolt period (Stage A and B), concomitant hardening of these layers occurs by the deposition of mineral salts therein. It is evident from the analyses of small pieces of the area of softening (Travis, 1955b) that the most abundant mineral constituent in the exoskeleton

is calcium. In order to determine what salts were deposited in the skeleton, further chemical analyses were carried out on the entire area of softening from late Stage C animals. The results of these analyses are indicated in Table I. From these results it is apparent that most of the calcium present in the skeleton is in the form of calcium carbonate. In order to determine whether this is deposited in the organic matrix of the skeleton as amorphous calcium carbonate or as crystalline aragonite or calcite, x-ray diffraction photographs (kindly made by Dr. C. Frondel, Department of Minerology, Harvard University), were taken of dry powder obtained from triturated pieces of the area of softening. These photographs (Fig. 3) indicate that calcium carbonate exists in the spiny lobster skeleton as calcite.

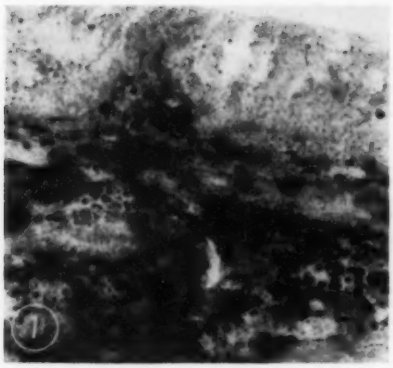
Since the basic organic components of the crustacean exoskeleton are chitin and protein, which are firmly associated with one another, Trim (1941), Stacy (1943) and Haworth (1946) regard the arthropod cuticle as a mucopolysaccharide because of the firm combination of carbohydrate-containing amino sugars (chitin) with the protein. Further, since calcium is the most abundant, if not the most important, mineral constituent within the crustacean skeleton, an emphasis will be placed on the abundance and distribution of glycogen, phosphatase, and calcium in the integumental tissues. These three constituents, among others, are of extreme importance in the development and calcification of the new skeleton.

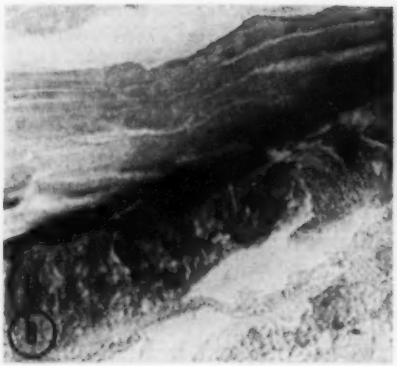
c. *Localization of glycogen, phosphatase and calcium*

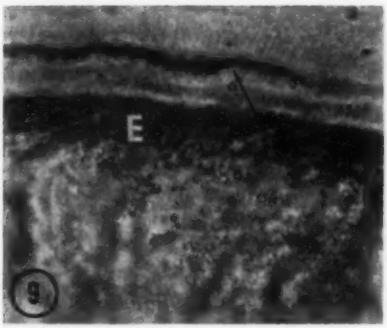
Glycogen: At *one day* following molt (Stage A), glycogen granules are scattered throughout the sub-epidermal connective tissue. Abundant amounts are concentrated at the bases of epidermal cells of the inner integument while the epidermal cells of the outer integument show little glycogen, which is localized in the proximal half of the cells (Fig. 4).

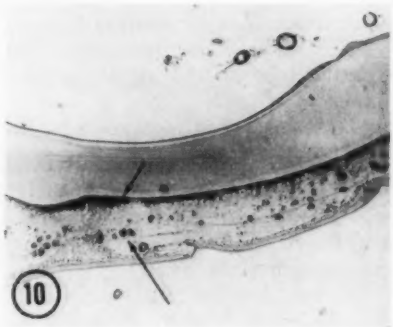
On the *second day* following molt, glycogen completely disappears from the inner epidermis (Fig. 5) and is not observed again in this tissue during the entire postmolt period. From two through four days following molt, little glycogen is apparent in the sub-epidermal connective tissues. Much heavier concentrations, on the other hand, are observed in the distal half of the outer epidermal cells (Fig. 6). By the *fifth day* glycogen accumulates in large amounts in the sub-epidermal tissues while the outer epidermis becomes almost depleted of it (Fig. 7). From the *sixth* through the *seventh day* most of the glycogen disappears from the sub-epidermal connective tissue and moves again to the outer epidermis where it is heavily concentrated in the distal portion of this tissue.

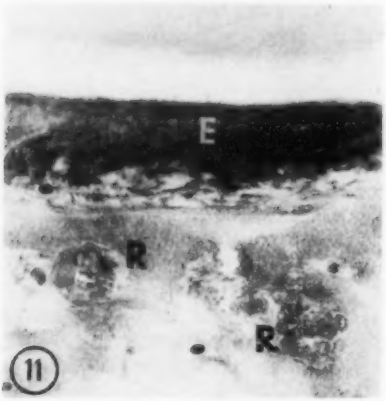
Phosphatase: Alkaline phosphatase becomes localized in the outer epidermis, being more heavily concentrated in the distal rather than the proximal half of the cells. Furthermore, the enzyme is heavily concentrated in the integument immediately above the epidermis (Fig. 8). It appears to be concentrated at this site in the region of the proximal portions of the innumerable pore canals. The localization of phosphatase in these sites is evident on the *first day following molt*, before calcification of the branchial integument begins, and remains in this localization throughout Stages A, B and very early C. In addition to its presence at these sites alkaline phosphatase is observed rather evenly distributed in the reserve cells during the entire period of observation. Control treated sections indicate that not only the enzyme but calcium as well are present in all of these sites from the second

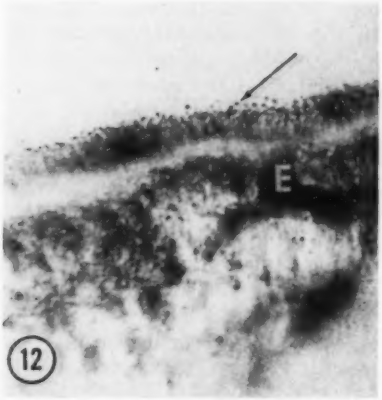












FIGS. 7–12.

through the eighth day, although purpurin and alizarin red S did not show the presence of calcium within the reserve cells before the third day following molt. It might also be pointed out that the enzyme is likewise heavily concentrated around newly developing bristles.

Calcium: Calcification of the new outer integument of the branchiostegites begins on the second day following molt (Stage B). Before calcification begins, however, the epidermis begins to concentrate calcium in markedly evident amounts (detectable by purpurin, alizarin red S, Von Kossa's method, Gomori's (1941) method for alkaline phosphatase and confirmed by microincineration). Before discussing the calcification of the skeleton the author would like to point out that of these methods used for the detection of calcium, Von Kossa's method and Gomori's (1941) method for alkaline phosphatase are the most useful for showing actual stages in calcification of the integument. Furthermore, these two methods show calcium salts or complexes in granular form.

At *one day* following molt and one day before calcification of the integument begins, the distal ends of the epidermal cells begin to show calcium. No reserve cells of the integumental tissues, as will be recalled, bind calcium at this time.

By the *second day*, the distal portions of the epidermal cells are filled with calcium. The tissue spaces or sinuses at the base of the epidermis and the blood channels, sometimes observed between the epidermal cells, show the presence of calcium. Although only a few reserve cells are apparent, a few show scattered granular deposits of calcium. At this same period a narrow band of calcium granules appears in the new principal layer immediately distal to, but paralleling, the epidermis (Fig. 9). Likewise, immediately underlying the epicuticle and within the pigmented zone a heavy concentration of rows of granules is observed.

By the *third day* the epidermal cells as well as the reserve cells of the sub-epidermal connective tissue are completely filled with calcium (Figs. 10, 11) detected by all methods used for this purpose. The heaviest concentrations of calcium granules in the epidermis are observed in the distal half of the cell. Here they are observed to be extruded from the distal ends of the epidermal cells in two distinct ways (Travis, 1951a, 1951c). As the post-exuvial layers are deposited, masses of cal-

FIGURE 7. At five days following molt glycogen has disappeared from the outer epidermis but is heavily concentrated by the sub-epidermal connective tissue. 760 ×.

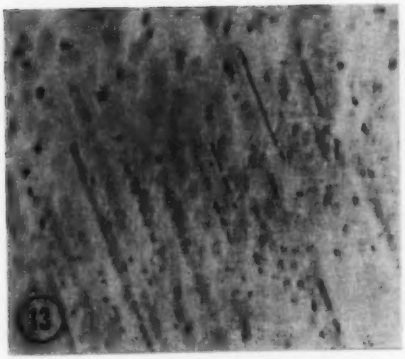
FIGURE 8. One day postmolt, before calcification begins in the branchial integument. Localization of alkaline phosphatase in the distal portion of the outer epidermis and in the integument immediately above the epidermis (region of the proximal portions of the innumerable pore canals, arrows). Phosphatase remains concentrated here through Stages A, B and early C. 420 ×.

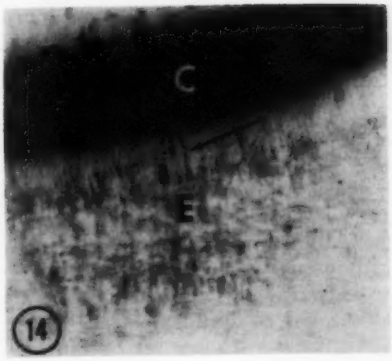
FIGURE 9. At two days postmolt calcification of the integument begins. Note that the distal portions of the outer epidermal cells (E) are filled with calcium and the narrow band of calcium granules (arrow) which appear in the newly forming principal layer immediately distal to, but paralleling, the epidermis. 2000 ×. Van Kossa's method.

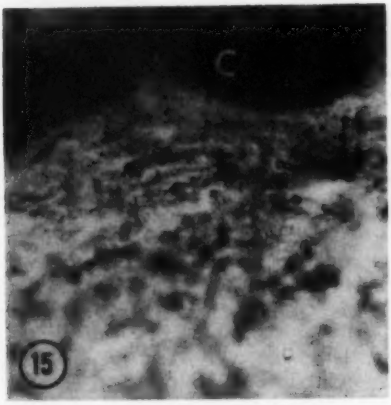
FIGURE 10. By the third day postmolt the epidermal cells (arrow) and the reserve cells (arrow) of the sub-epidermal connective tissue are filled with calcium. 90 ×.

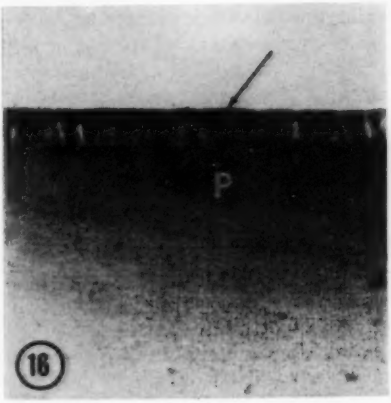
FIGURE 11. Higher magnification showing that the epidermal cells (E) and reserve cells (R) of sub-epidermal connective tissues, at three days postmolt, are filled with calcium. Note lumpy stainable balls of material in the reserve cells. 760 ×. Alizarin red S staining.

FIGURE 12. As the post-exuvial layers are deposited (three days following molt) masses of calcium granules are simultaneously extruded from the epidermal cells (E) to form narrow calcified bands paralleling the epidermis (arrow). 2000 ×. Van Kossa's method.









FIGS. 13–18.

cium granules are simultaneously extruded to form narrow bands paralleling the epidermis (Fig. 12). In the pre-exuvial layers of the skeleton, deposited before molt, calcium granules are observed in uniform vertical rows. These rows of granules are particularly evident in the pigmented layer (Fig. 13) and can be seen to emanate from the epidermis (Fig. 14). The rows of granules correspond in location to the pore canals or vertical striae. Calcification of the pre-exuvial layers occurs after their formation. Hence, calcium has to be transported and deposited some distance from the epidermis. This transport, therefore, appears to occur through the pore canals.

From *four through seven days* calcium in the epidermis remains equal in amount to that observed on the third day.

Calcification continues to occur as the post-exuvial layers are deposited. As a consequence, the horizontal bands of granules paralleling the epidermis have thickened considerably by the end of Stage B (Fig. 15). By the *seventh day* portions of the inner integument are completely calcified. Calcification of the inner integument appears to occur in an identical fashion to that observed in the pre-exuvial layers of the outer integument.

2. *The hepatopancreas*

a. *Tissues*

The hepatopancreas, as discussed by Travis (1955a), is the major storage depot of organic and mineral reserves during Stage D and is, consequently, the major organ from which these reserves are mobilized when needed by other tissues during the postmolt period.

For five days following molt (Stage A and part of Stage B), the epithelial tissue of the hepatopancreatic tubules is predominantly of the absorbing type. This epithelial tissue consists of long tall columnar cells without large vacuoles and which may have either a central or basal nucleus (the so-called B_1 or R cells of Hirsch and Jacobs, 1928, 1930) as discussed by Travis (1955a). A few of the epithelial cells are of the secretory type (the vesicular or B_2 cells of Hirsch and Jacobs). These are enlarged swollen cells which enclose large vacuoles, some of

FIGURE 13. In the pre-exuvial layers of the skeleton, deposited before molt, calcium granules are observed in uniform vertical rows corresponding in location to the pore canals. 2000 ×. Von Kossa's method.

FIGURE 14. Note narrow vertical rows of calcium granules, corresponding in location to the pore canals (arrow) emanating from the epidermis (E). Also note the rather wide band of calcium granules (C) in a newly formed portion of the principal zone paralleling the epidermis. 2000 ×. Von Kossa's method.

FIGURE 15. Horizontal bands of calcium granules paralleling the epidermis (E) continue to be deposited as the post-exuvial layers are deposited and a thick calcified band (C) in the new principal layer is noted by the end of Stage B. 2000 ×. Von Kossa's method.

FIGURE 16. By seven days following molt (end of Stage B) the epicuticle (arrow) and most of the pigmented layer (P) appear to be fully calcified except in certain areas where this is not complete. 2000 ×. Von Kossa's method.

FIGURE 17. Note the heavy concentration of glycogen at the basal and distal ends of the absorption cells and at the periphery of the vacuoles of secretory cells in the tubular epithelium of the hepatopancreas. One day following molt (Stage A). 200 ×.

FIGURE 18. At seven days following molt (end of Stage B) the tubular epithelium of the hepatopancreas is virtually devoid of glycogen. 200 ×.

which contain stainable material. These cells (Travis, 1955a) undergo apocrine breakdown in *Panulirus*. Their vacuolar contents plus adjacent cytoplasm are discharged into the lumen of the hepatopancreatic tubules, leaving only the basal region and nucleus of the cell intact. However, none of these observed secretory cells, during this period of five days following molt, show any sign of apocrine breakdown. This would be expected because the animals are still undergoing a period of inanition (Travis, 1954, 1955a, 1955b). Stainable material in the large vacuoles of the secretory cells frequently indicates the presence of lipid and mucopolysaccharide.

By the sixth and seventh day following molt, the predominant cell types observed in the epithelial tissue of the tubules are the secretory cells. On the seventh day there is much evidence of apocrine secretion within these cells. In general, most animals begin to feed again on the seventh day. This would not only account for the numerous secretory cells but also for the apocrine breakdown of many of these cells.

The large oval reserve cells are likewise apparent in the connective tissue between the individual tubules of the hepatopancreas. They similarly show, as do the reserve cells of the integumental tissues, the presence of phosphatase, mucopolysaccharide and calcium. Much lipid is also present during Stage A and B. From the fifth through the seventh day the reserve cells of the hepatopancreas show the presence of large vacuoles (Fig. 2), some of which contain flaky or granular-like stainable material while others appear to be clear. This is possibly correlated with a decrease in mucopolysaccharide, lipid, and calcium content in these cells. The reserve cells of the hepatopancreas, like those of the integumental tissues, undergo cyclic peaks and declines in size and abundance, changes in structural appearance, and the storing and apparent release of reserves as indicated below.

During the *intermolt period* (late Stage C) they are numerous and large, ranging in size from 17–38 μ with an average size of 30 μ. They show markedly evident amounts of mucopolysaccharide. A similar situation prevails during the *premolt period*. The cells range in size from 17–38 μ with an average size of 32 μ and show large stainable spheres of material. At both of these stages much mucopolysaccharide is present. Calcium is likewise apparent in large amounts in the hepatopancreatic reserve cells, while the integumental tissue reserve cells are devoid of it during these periods.

By the *first day* following molt, there are few reserve cells present in the hepatopancreas and these have undergone a general decrease in size (8–29 μ, average size 16 μ), a situation opposite to that observed in the integumental tissues. Of the reserves, however, little calcium seems to be apparent and there is no evidence of spheres of material found within them. On the *second day* (beginning of Stage B) the cells remain few in number but have increased in size (range 24–38 μ, average size 31 μ), again a situation opposite to that observed in the integumental tissues, although no large spheres of material are evident. They again show the presence of small amounts of calcium. At *three days*, as in the integumental tissues, the reserve cells become numerous and remain large (range 24–48 μ, average size 30 μ). They take on a distinct "mulberry" appearance and show the presence of mucopolysaccharide and markedly evident amounts of calcium. A similar situation prevails on the *fourth day*. The cells in the hepatopancreas remain numerous and large (range 26–43 μ, average size 35 μ) and likewise show abundant mucopolysaccharide

and calcium. By the *fifth day* few cells are apparent and they are slightly smaller in size (21–48 μ, average size 28 μ). These cells become highly vacuolated, although some balls of material are present within the vacuoles, and show only a slightly pink coloration with PAS, a condition similar to that observed in the reserve cells of the integumental tissues. Little calcium is apparent. Except for the increase in number and slight increase in size (29–38 μ, average size 32 μ) the reserve cells on the *sixth day* remain similar to the *fifth day* condition though they contain large single vacuoles with no stainable balls of material. They remain numerous on the *seventh day* but the average size is slightly smaller (24–32 μ, average size 29 μ). Except for these changes they are similar to the *fifth day* condition with respect to reserves. It should be added that at all stages of the observation period, phosphatase and lipid are present.

Since different groups of animals were used for the study of hepatopancreatic and integumental tissues (fixed in different years), it is difficult to determine whether or not the cycles may be slightly out of phase at the sites of observation. The information at hand (Sewell, 1955; Travis, 1955a) strongly suggests that much more information is needed on these highly interesting and obviously important reserve cells.

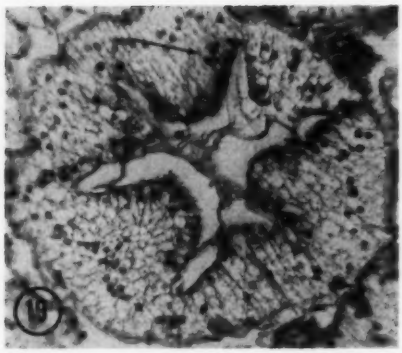
b. *Localization of glycogen, phosphatase, calcium and lipid*

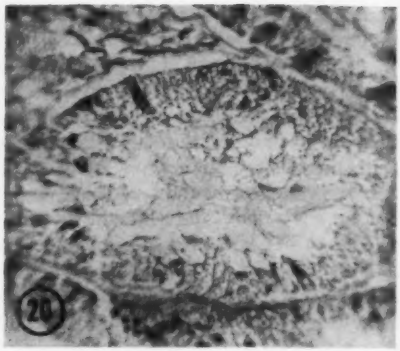
Glycogen: Although the concentration of glycogen within the tubular tissue on the first day following molt (Fig. 17) would compare with the premolt condition (see Figure 30; Travis, 1955a), there is a progressive decrease in abundance from the first through the seventh day. A decidedly marked decrease occurs by the sixth day and by the seventh day hardly a single granule of free glycogen can be detected within the tubular tissue (Fig. 18). During Stages A and B, glycogen is more heavily distributed in the distal and basal ends of the absorption cells. When mature secretory cells are observed during the early phase of Stage B, glycogen granules are localized at the periphery of the vacuoles and are sometimes observed within the lumen of the tubules.

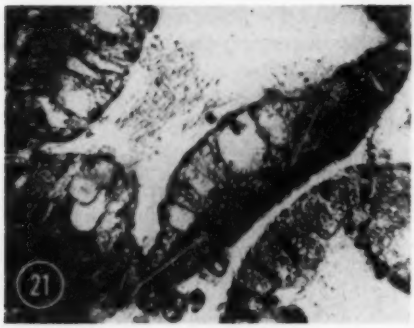
The disappearance of glycogen from the hepatopancreas by the seventh day might be expected because of the need of this constituent in the synthesis of the new skeleton. The integumental tissues, among others, therefore, accumulate and use large amounts of glycogen at the expense of the hepatopancreas. This is evident in both the premolt and postmolt period.

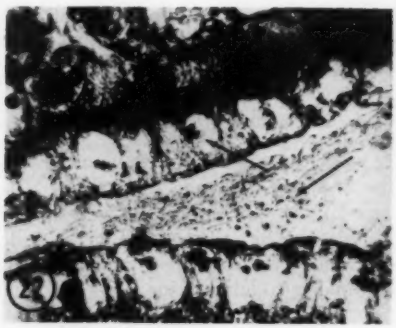
Phosphatase: During the early postmolt period alkaline phosphatase is almost absent from the striated borders of the tubular tissue but remains localized around the calcospherites (Fig. 19), which disappear progressively as calcification of the skeleton occurs. From the sixth through the seventh day when many large secretory cells are present, the enzyme is localized around the periphery of small and large secretory vacuoles. It is at all times present in the reserve cells.

Calcium phosphate: As in the premolt animals, the tubular epithelium of the postmolt animal is marked by the presence of innumerable calcospherites in the apical ends of the absorbing cells, very few being apparent in the small number of secretory cells. In the postmolt animal, however, these calcospherites disappear progressively as the skeleton is calcified. They have markedly decreased by the fifth day and by the seventh day (Fig. 20) hardly a single calcospherite can be de-









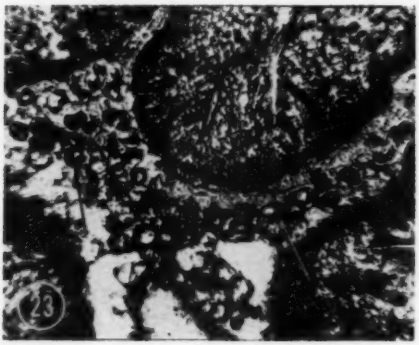


FIGURE 19. The tubular epithelium of the hepatopancreas at one day following molt (Stage A). Note alkaline phosphatase localized around innumerable calcospherites (arrow) at the apical ends of absorbing cells. 200 ×.

FIGURE 20. At seven days following molt, note that there is almost a complete absence of calcospherites and the enzyme alkaline phosphatase in the tubular epithelium of the hepatopancreas, a situation that occurs progressively as the integument is calcified. 160 ×.

tected. The complete disappearance of the calcospherites by the seventh day would be expected in *Panulirus* because these animals have to rely completely on this stored phosphate, stockpiled during the two-week premolt inanition period, as a source of phosphate for incorporation into the new skeleton. Since, as was pointed out previously (Travis, 1954, 1955a, 1955b), phosphate is obtained primarily from food, the stockpile of stored phosphate in the hepatopancreas would readily be depleted by the sixth or seventh day of starvation following molt. From low blood values following molt (Travis, 1955b) it is evident that the normal intermolt blood concentration within the body would not be replenished for at least three or four weeks following molt. Although the reserve cells of the hepatopancreas show the presence of calcium from one through seven days following molt, from the fifth through the seventh day little is apparent.

Lipid: For the entire observation period (Stages A and B) droplets of lipid are found throughout the epithelial tissue of the hepatopancreatic tubules. There would appear, by the sixth and seventh day, to be a decrease over that observed in the premolt animal, although much is still apparent. When secretory cells are apparent in great numbers (6 and 7 days) lipid droplets are frequently observed within the vacuoles (Figs. 21, 22). On the seventh day, when apocrine breakdown is evident, and when some of the animals begin to feed a little, lipid material becomes quite apparent within the lumen of the tubules, a condition which would be correlated with extracellular digestion of this constituent, as Van Weel (1955) has shown.

The reserve cells contain considerable quantities of lipid for the entire postmolt observation period (Fig. 23). There does appear to be a decrease over that observed during the intermolt (late Stage C) and the premolt period. Because of the presence of considerable quantities of lipid within the hepatopancreas at this time, a histochemical or a qualitative difference in amount is difficult, with certainty, to detect.

Discussion

The continued accretionary growth and hardening of the post-exuvial layers of the skeleton imposes upon the epidermis two major tasks, namely the synthesis and elaboration of the organic matrix and the simultaneous or accompanying elaboration of constituents for hardening the skeleton which may or may not alter the properties of the basic organic components, chitin and protein. The complexities of these two functions cannot be over-emphasized. Although the epidermis takes the lead in the performance of these tasks, the importance of other tissues, such as the hepatopancreatic and subepidermal tissues, cannot be under-estimated.

As the principal layer of the skeleton in *Panulirus* is deposited during the early postmolt period of observation (Stages A and B), the outer epidermis shows, tinctorially, that considerable amounts of a glyco- or mucoprotein are concentrated or

Figure 21. The distribution of lipid droplets within secretory cells of the hepatopancreas (arrows). The secretory cells become apparent in great numbers at six and seven days following molt. 200 ×.

Figure 22. Seven days following molt. Note lipid droplets (arrow) within the vacuoles of the secretory cells and within the lumen of the tubules (arrow). 200 ×.

Figure 23. Note that considerable quantities of lipid are bound by the reserve cells (arrows) of the hepatopancreas. 100 ×.

synthesized by this tissue. Similarly, during Stage A and most of B, the pigmented layer, one of the two pre-exuvial layers formed before molt, shows the presence of this same mucopolysaccharide. By the fifth day the pigmented layer no longer indicates a positive reaction for this constituent. There is a tinctorial change from gamma metachromasia (pink-purple) to beta metachromasia (deep purple) with toluidine blue, which indicates that the properties of the basic organic components, chitin and protein, have been altered. Possibly this is caused by considerable impregnation with calcium salts at this time or, more certainly, by quinones which form cross-linkages with the native protein phase of the cuticle (Pryor, 1940). The net result of this combination is the formation of a highly stable and insoluble product. As Krishnan (1951) pointed out, the tanning by quinones of the pigmented layer occurs in *Carcinus maenas* shortly following molt and this is followed by pigmentation at a slightly later period. It is, therefore, possible that both of these related processes are completed by the fifth day in *Panulirus,* thus causing this change in tinctorial properties.

The presence of muco- or glycoprotein in the epidermis for the entire postmolt observation period (Stages A and B) is doubtlessly related to the secretion and development of the principal layer, which is the only post-exuvial layer deposited during this period. This layer, like the pre-exuvial pigmented layer and post-exuvial membranous layer (formed and completed from the third through the fourth week following molt in *Panulirus*), consists as in insects of the basic organic components, chitin and protein, which are closely associated with one another. The firm combination of these two organic constituents has led Trim (1941), Stacy (1943) and Haworth (1946) to regard the arthropod cuticle as a mucopolysaccharide because of the firm combination of the carbohydrate-containing amino sugars (chitin) with the protein. Richards (1951) has pointed out that the consideration of the arthropod cuticle as a double set of layers (the outer set being composed of lipoprotein and the inner set being composed of glyco- or mucoproteins) is advantageous. This consideration emphasizes that the major cuticular components seem to be formed and secreted as conjugated proteins and not as separate components.

The principal layer in *Panulirus* always shows, tinctorially, the presence of muco- or glycoproteins with the exception of the fact that immediately after each layer is deposited a green, rather than a pink, coloration with toluidine blue occurs. This suggests that the reactive groups are slightly altered following their immediate formation. Although the early stages in the formation of the membranous layer were not followed, the completed layer, in contrast to the principal layer, shows a green rather than a pink coloration, which indicates that this layer is, in some way, different from the principal layer. This is further revealed by the fact that calcium salts are never bound in this layer.

During the deposition of the principal layer of the outer integument glycogen accumulates in large amounts in the epidermis. There is a periodic shift of glycogen from the sub-epidermal tissues to the outer epidermis (Travis, 1951a, 1951c, 1955a) and in turn a shift from the hepatopancreatic tissues to these integumental tissues (see Observations). During the postmolt period of accumulation and utilization of glycogen by the integumental tissues (Stages A and B), hepatopancreatic glycogen progressively disappears. Although it is abundant in the tubular tissue on the first day following molt, there is a marked decrease in glycogen by the

fifth day and hardly a single granule of free glycogen is present by the seventh day. The rhythmical accumulation by, and disappearance of, glycogen from the sub-epidermal tissues and similarly its accumulation by the epidermal cells and disappearance from the sub-epidermal tissues suggests a rhythmical cycle of accumulation and utilization in the epidermis, at the expense of sub-epidermal tissue glycogen. Likewise, the rhythmical accumulation of glycogen by the sub-epidermal tissue and progressive disappearance from the hepatopancreas similarly suggests that there are marked cycles of accumulation and utilization. These cycles of accumulation and utilization stem from the epidermis, which takes the lead in the elaboration of the post-exuvial layers of the integument, but also involves the hepatopancreas, which serves as the major storage organ from which such reserves can be mobilized and upon which the epidermis is ultimately dependent for the successful completion of its tasks. Since feeding begins on the seventh day following molt in the summer months, a constant supply of glycogen would be available to the epidermis until the integument is completed (late Stage C), but is not stockpiled in the hepatopancreas again until Stage D. The stockpiling of glycogen during this period of inanition would suggest strongly that the source of this constituent is from the large quantities of lipid reserves, likewise present in the hepatopancreas at this time. By the conversion of some lipid, through its glycerol moiety, to carbohydrate, the latter being stored as glycogen, the peak glycogen concentration could be achieved during Stage D. As was pointed out by Travis (1955a), evidence suggests (Renaud, 1949) that during periods of inanition (Stages D, A and B), lipids likewise serve as a major source of energy by playing a principal role in oxidative metabolism.

The periodic accumulation and utilization of glycogen by the epidermis as the post-exuvial layers are deposited suggests that glycogen is a necessary precursor for chitin formation. This possibility, as discussed by Travis (1955a), has been suggested by Verne (1924, 1926), Mataczyńska-Suchcitz (1948), Renaud (1949), Travis (1951a, 1955a) and Schwabe *et al.* (1952). Glycogen may likewise serve as a ready energy-source for the synthesis and elaboration of the organic constituents of the integument. This possibility has been suggested by Bradfield (1951). He found an abundance of glycogen in regenerating epidermis of the vertebrates. As the outermost cells keratinized, glycogen disappeared. He attributed this disappearance to the utilization of glycogen for the supply of energy in keratin synthesis. Glycogen may further serve indirectly as added substrate for phosphatase action after its hydrolysis and phosphorylation by phosphorylases. In this way, it has been postulated as one of the necessary mechanisms in calcification of bone and teeth of vertebrates (Robison and Soames, 1924; Harris, 1932; Glock, 1940; Horowitz, 1942; Engel, 1948; Marks and Shorr, 1950 and others). It is more likely, however, that glycogen participates in all of these functions and possibly others that have not been mentioned.

During the entire postmolt observation period (Stage A and D), alkaline phosphatase is heavily concentrated in the distal ends of the outer epidermal cells and is observed in the integument immediately distal to but paralleling the epidermis. The localization of the enzyme in this latter site is distinctly apparent by the first day following molt before any calcification begins in the branchial region of the integument. Krugler and Birkner (1948) noted a similar localization of the enzyme in the integument of the crayfish during premolt. In *Panulirus* this is a

strategic location for the enzyme during the postmolt period because it is in a region of high activity as the deposition and hardening of the post-exuvial layers occur. Further, it would appear that the enzyme may be specifically localized in the proximal portions of the pore canals. Because of its heavy concentration along this entire region of the integument, however, its specific localization in the pore canals is difficult to determine with certainty. The enzyme is likewise heavily concentrated in the integument around newly developing bristles. In the sub-epidermal tissues it is observed in the reserve cells.

In the hepatopancreas the most marked localization of the enzyme is seen around the innumerable calcospherites in the distal portions of the absorption cells. The enzyme is observed in the striated borders of these cells and on the sixth and seventh day, when a predominance of secretory cells is evident, it is observed at the periphery of small and large vacuoles. The reserve cells within the blood or tissue spaces between the tubules of the hepatopancreas likewise show the presence of the enzyme.

As in the premolt animal, phosphatase is localized around the calcospherites in the absorption cells of the hepatopancreas and since these disappear progressively as calcification of the post-exuvial layers occurs, it is possible that the enzyme participates in the mobilization of this reserve for transfer to the integument. It may do so by dephosphorylating, in some way, the precipitated complex. In this role, it would be serving in resorption at this site and could at the same time be involved in mediating the synthesis of other phosphoric esters to be conveyed via the blood from the hepatopancreas to the integumental tissues. That this indeed may be an important function of phosphatase in bone resorption has been suggested by McLean and Urist (1955). Further, its localization around the periphery of small and large vacuoles on the sixth and seventh day would suggest that the enzyme is possibly involved, in some way, with the synthesis of secretory products or the transfer of these products from the adjacent cytoplasm into the secretory vacuoles. Phosphatase localization at the striated borders of the absorption, as well as the secretory, cells would suggest that when these cells are active the enzyme would likewise serve the function of participating in transfer reactions by producing molecules which enter or leave the cells more readily. Such a function has been suggested by Moog (1946).

The concentration of the enzyme at the distal ends of the outer epidermal cells and in the integument immediately distal to, but paralleling, the epidermis suggests its extremely important functions in the deposition and hardening of the post-exuvial layers. The periodic accumulation and utilization of glycogen by the epidermis, as was pointed out earlier, would suggest that possibly this constituent, glycogen, is used as a precursor in chitin formation. If this is so, and if the synthesis of chitin occurs, as Renaud (1949) suggested, by the hydrolysis of glycogen and dephosphorylation of glucose phosphate to glucose, this step being followed by subsequent steps to yield chitin, phosphatase would play an important role in this chain of events by its dephosphorylation of glucose phosphate to glucose, a possible starting point for chitin formation. Likewise, if glycogen were used as an energy-source for the synthesis and elaboration of the organic matrix, phosphatase would be intimately involved in these reactions. Glycogen, as suggested earlier, could serve indirectly as added substrate for phosphatase action. The distribution of phosphatase and mucopolysaccharide in the epidermis and its distribution in the

region of the newly forming post-exuvial layers of the integument, immediately above the epidermis, suggests that it may play a very important part in the formation of the ground substance (mucopolysaccharide) of the post-exuvial layers. Furthermore, the enzyme is thought to play an important role in the manufacture of fibrous proteins, thus participating in the formation of the ground substance of bone (McLean and Urist, 1955). Moog and Wenger (1952), however, have suggested that since the enzyme and mucopolysaccharide are frequently found together in fibrous structures, the mucopolysaccharide constitutes part of a cytoskeletal mechanism to which the enzyme is bound.

The appearance of alkaline phosphatase in the integument immediately distal to and paralleling the epidermis one day before calcification begins, likewise suggests to the author that the enzyme is intimately involved in calcification of the integument. In such a localization it could provide a mechanism for the production of a local high concentration of phosphate ions. In the presence of calcium ions, transferred across the cell membranes of the epidermis, phosphate could then unite to form the calcium salt, calcium phosphate, which constitutes about 3% of the total

TABLE I

Analyses to indicate the amount of mineral and organic matter in the entire area of softening of a late Stage C animal

Substance analyzed	Per cent present in the area of softening	Calculated % of salts present	
CaO	24.64	$CaCO_3$	42.39
MgO	1.98	$MgCO_3$	4.04
P_2O_5	1.40	$Ca_3(PO_4)_2$	3.05
CO_2	21.39	Carbonates unaccounted for	2.22
Per cent mineral as Ca, P, and Mg oxides	28.02		
Per cent organic matter	71.98		

mineral salt of the integument (Table I). The almost certain presence of phosphorylases at these sites, although not specifically determined, would likewise be expected to be important in the calcification of the integument, by synthesizing potential substrates for phosphatase action in zones of calcification.

Hardening of the crustacean skeleton occurs by quinone tanning and calcification. Hardening by quinones is a result of the oxidation of polyphenols to quinones, which form cross linkages with the native protein of the cuticle. The net result of this combination is a highly stable and highly insoluble product. Of the pre-exuvial layers, the epicuticle of *Carcinus maenas* is hardened by quinones shortly after its formation, whereas subsequent hardening of the pigmented layer occurs soon after molt (Krishnan, 1951). As was suggested in a previous section of the discussion, changes in tinctorial properties of the pigmented layer suggest that the process of hardening by quinones is complete by the fifth day following molt. Quinone tanning, although the primary cause of hardening in the exoskeleton of insects, plays a much smaller role in Crustacea (Dennell, 1947), calcification being the major cause of hardening.

Calcification begins on the second day following molt in *Panulirus* and occurs thereafter simultaneously with or immediately accompanying the elaboration of layers of the principal zone. Further, the additional task posed to the epidermis is that of calcifying the *pre-exuvial layers;* calcification, in this case, is of course a process that is accomplished long after their formation but during the same time at which the post-exuvial layers are being calcified.

In the distal region of the epidermal cells, where calcium becomes most heavily concentrated, extrusion of calcium from this tissue occurs in two distinct ways (Travis, 1951a, 1951c). As the post-exuvial layers are deposited, masses of calcium granules are simultaneously extruded, thus forming narrow bands paralleling the epidermis. In the pre-exuvial layers, on the other hand, calcium granules are observed in uniform vertical rows. These rows, as pointed out earlier, are particularly evident in the pigmented layer, and are likewise observed to emanate from the epidermis. They correspond in location to the pore canals or vertical striae, protoplasmic extensions of the epidermis. Thus, in the case of the pre-exuvial layers, calcification occurs after their formation. Hence, calcium must be transported and deposited some distance from the newly forming pre-exuvial layers. This transport occurs through the pore canals, thus enabling the epidermis to act at these distant sites.

Calcification of the integument continues and is almost entirely completed by the seventh day (end of Stage B) in the epicuticle and most of the pigmented layer.

While calcification of the integument occurs, the reserve cells in the subepidermal tissues undergo what appear to be cyclic peaks in calcium storage, possibly alternating with cyclic release to the epidermis. If calcium in these cells is used periodically by the epidermis, which it probably is, the reserve cells could serve as reservoirs for providing additional calcium during periods of concentration by the epidermis. At no time during Stage A and B, however, are the epidermal cells depleted of calcium. This might be expected because the concentration of calcium in the blood (Travis, 1951b, 1955b) is sufficiently high to provide a continued supply of this element to the epidermis. Previous reference to the reserve cells has already been made as to their cyclic peaks and declines in abundance and size, change in structural appearance and in the binding and release of reserves, other than calcium (see Observations) and will not therefore be discussed in this section.

As calcification of the integument occurs there is a progressive decrease in number of calcospherites—spherules of calcium phosphate—present in the absorption cells of the hepatopancreas. Though abundant on the first day following molt they progressively decrease in number as calcification of the new integument occurs and by the seventh day hardly a single calcospherite is to be detected. The calcospherites, premolt storage depots of reserve phosphate from the old skeleton (Travis, 1955a), represent a major source, therefore, from which phosphate can be mobilized for hardening of the new skeleton during Stage B, a time at which the animals do not feed. Since the spiny lobster obtains most of its phosphorus from food and since the animals do not feed for two weeks before molt, when resorbed phosphorus from the old skeleton is being stockpiled in the hepatopancreas (Travis, 1955a), one would expect a depletion of this mineral reserve during the first week following molt as calcification of the new integument occurs. This depletion is also evidenced by the low blood-phosphorus levels following molt (Travis, 1955b).

It is interesting that the reserve cells of the hepatopancreas show the presence

of calcium from one through seven days following molt but from the fifth through the seventh day little is apparent. The fifth through the seventh day is a period when these cells become vacuolated, which may be correlated with the apparent decrease in mucopolysaccharide, lipid and calcium. It is possible that as the calcium phosphate is mobilized from the calcospherites it is immediately transferred from the absorption cells to the reserve cells and from these to carriers in the blood, possibly organic acids of oxidative and glycolytic metabolism. The evidence at hand, however, is not sufficient at this time to determine whether there is an actual movement of the reserve cells from the hepatopancreatic tissues to the integumental tissues.

Little has been said about the development and hardening of the inner integument during Stages A and B. It will be recalled that glycogen is observed in abundance at the bases of the epidermal cells of the inner integument on the first day following molt (Stage A) but completely disappears from the inner epidermis by the second day, not to be observed again at this site for the entire observation period. Similarly, no further thickening of this integument occurs after the second day following molt, indicating that the development of the inner integument of the branchiostegites is completed during a period of three days preceding molt and two days following molt (Travis, 1955a). This does not mean, however, that hardening by calcification is completed at this time. Calcification of the inner integument, as in the pre-exuvial layers of the outer integument, occurs after its formation. By the fourth day the uniformly staining endocuticle of the inner integument has begun to calcify and by the seventh day portions of it have completely calcified. Calcification in the inner integument occurs in the same fashion as that observed in the pre-exuvial layers of the outer integument, *i.e.*, via the port canals.

The two major tasks which must be achieved by the epidermis, namely, the synthesis and elaboration of an organic matrix and the simultaneous or accompanying elaboration of calcium salts for hardening of the newly developing skeleton, are not completely accomplished before the fourth week following molt during the summer months. Calcification is probably completed by the third week following molt, a time at which the membranous or non-calcified layer begins to be formed. This layer is not fully completed before the fourth week following molt (late Stage C or C_4 of Drach, 1939). Approximately three weeks following the completion of the integument the epidermis is again confronted with the preparation for growth in size of the animal (Travis, 1955a).

The cells of the epidermis, like the osteoblasts of bone, synthesize and elaborate the organic matrix of the skeleton and, unlike the osteoblasts of bone, they actually concentrate and secrete the mineral constituents, principally calcium, which are precipitated in the matrix. Furthermore, the epidermis, like the osteoclasts of bone, participates intimately in processes of resorption of the integument. It elaborates the proteinases and chitinases which break down the organic matrix. It likewise resorbs these organic breakdown products along with the mineral constituents and also participates in their transfer across its cell membranes to the blood for further handling (Travis, 1955b).

Following molt the transfer of calcium ions from the blood across the cell membranes of the epidermis and the concentration of calcium by this tissue is truly remarkable. Within the epidermal cells, the calcium is doubtlessly immobilized ionically by the binding capacity of weakly acidic groups of protein, succinate, lactate, bicarbonate, phosphate, citrate or by other anionic groups. On release of calcium

to the exterior of the cell, an alteration in the binding capacity of anionic groups is necessary. After release from the epidermis, calcium is precipitated as salts, by various mechanisms, in the organic matrix of the integument.

The distribution of alkaline phosphatase in the distal portion of the epidermis and particularly in the integument immediately distal to and paralleling the epidermis suggests that this enzyme would provide a mechanism for the local high concentration of phosphate ions, which in the presence of some of the calcium released from the epidermis could account for the precipitation of the 3% calcium phosphate of the integument (Table I).

Calcium carbonate, however, is the principal salt of the spiny lobster skeleton, and Crustacea in general, and constitutes approximately 42% of the total mineral deposited in the skeleton of *Panulirus*. It is, therefore, of interest to point out a related and possibly important enzyme involved in calcification of the skeleton. This is the enzyme, carbonic anhydrase. Sabotka and Kann (1941) found that this enzyme was not present in the gills of *Panulirus argus*. Because of this, they suggested that elimination of CO_2 is not confined to the gills, but that the bicarbonate formed may be eliminated in the skeleton by precipitation as $CaCO_3$. Maluf (1940) found that considerable quantities of carbonic anhydrase were present in the epidermis and skeleton of the crayfish, *Cambarus clarkii* and the American lobster, *Homarus americanus*. The fact that alkaline phosphatase is found in the distal ends of the epidermal cells and integument immediately above and paralleling the epidermis would suggest that the hydrogen ion concentration is low and the pH is on the alkaline side (between 8–10). At this pH, dissociation of bicarbonate into $CO_3^=$ and H^+ would be expected. In the presence of calcium ions released from the epidermis, the precipitation of calcium carbonate could occur. Thus, again the mechanism and conditions exist for the production of local high concentrations of bicarbonate and carbonate ions in the epidermis and integument of Crustacea.

The manner in which calcium carbonate is precipitated, *i.e.*, as calcite rather than aragonite or amorphous calcium carbonate, is undoubtedly determined by conditions inherent in the organic matrix which favor calcite precipitation. Prenant (1927), however, pointed out that the condition determining the state of calcium carbonate precipitation is the proportion of phosphates to carbonates, as indicated by the P_2O_5/CO_2 ratio. If the ratio is more than 0.015, calcium carbonate is deposited in amorphous form. If the ratio is 0.105 or less, the calcium is deposited in crystalline form. The P_2O_5/CO_2 ratio in *Panulirus* is 0.0657. Calcium carbonate in *Panulirus* is precipitated as calcite, which is consistent with the idea proposed by Prenant. This would not mean, however, that conditions in the organic matrix do not favor calcite, rather than aragonite, precipitation.

That the calcification mechanism in *Panulirus* may be influenced by the presence of organic acids such as lactic, succinic, and citric acid at the sites of calcification has yet to be investigated. These acids have a marked propensity for forming weakly ionized salts with calcium. It is highly likely, therefore, that, as in the calcification of bone, citric acid plays an important role in the calcification mechanism of Crustacea. It is known from the work of Dickens (1941) that more than 70% of the citric acid of the human body is in the skeleton and that as much as 1% of the fresh weight of bone may be accounted for as citrate. It is not definitely known whether citrate is present as an ion or precipitated as a calcium citrate complex. Bones examined for enzymes of the citric acid cycle have shown that by comparison

with other tissues, such as the kidney or liver, citrogenase and aconitase activities are much greater than those of isocitric dehydrogenase (McLean and Urist, 1955). As these authors point out, the mechanism for the production of local high concentrations of citric acid exists in bone. Other than the work of Thunberg (1949), however, as quoted from Thunberg (1953) and Steinhardt (1946), nothing has been done with the role of citric acid in the calcification process of the Crustacea. Thunberg found that at least 0.8% of the gastroliths of a European crayfish was constituted of citric acid. This would suggest strongly that citric acid is involved, as in bone, in some way in the calcification process. Steinhardt (1946) has further pointed out that in structures such as bone and gastroliths, where high concentrations of citric acid are found, the phosphorus and calcium content is also high. In such cases, citrate probably exists in a complex form in which calcium, phosphoric and citric acid enter.

It is highly likely, therefore, that within the integument of Crustacea, as in the gastroliths of the crayfish, the mechanism for the production of local high concentrations of citric acid exists. If so, the citrate produced, having a marked propensity for forming weakly ionized salts with certain cations such as calcium, could combine with calcium released from the epidermis and could enter into the mineral complex of calcium salts of the integument. If this is the case, increases and decreases of citrate formed enzymatically within tissues may be one of the regulators of ionic calcium activity. Furthermore, in normal metabolism, the normal activity of tissues may be controlled, in part, by the interaction of ionic calcium with citrate reached via the tricarboxylic cycle. Such a possibility has been suggested for the mammal by Peters (1950).

It is apparent from the foregoing discussion that the continued growth of the skeleton in an accretionary manner and the hardening of it by calcification, two of the major tasks confronting the crustacean from Stage A to late Stage C, are indeed complex. Although these extraordinary duties are put to the epidermis, which takes the lead in the performance of them, the importance of the other tissues, namely the hepatopancreatic and sub-epidermal tissues, should not be under-estimated.

Summary

1. During the early postmolt period (Stages A and B) as rapid accretionary growth and calcification of the skeleton are occurring, changes are observed in the hepatopancreas and integumental tissues.

2. As the principal layer of the skeleton in *Panulirus* is deposited during Stage A and B, the outer epidermis concentrates or synthesizes a considerable amount of glyco- or mucoprotein, which is probably related to and involved in the secretion and development of this layer. Similarly, the pigmented layer, one of the preexuvial layers formed before molt, shows the presence of this same mucopolysaccharide. Near the end of Stage B, however, the properties of the basic organic components in the pigmented layer have been altered, possibly by considerable impregnation with calcium salts or by quinones.

3. During the deposition of the principal layer of the outer integument, glycogen accumulates in large amounts in the epidermis. There is a periodic shift of glycogen from the sub-epidermal tissues to the outer epidermis and in turn a shift from the hepatopancreatic tissues to the integumental tissues. During this period

of accumulation and utilization by the integumental tissues (Stages A and B), hepatopancreatic glycogen progressively disappears and by the end of Stage B none remains.

4. The possibility of lipid conversion to carbohydrate, and the storing of this as glycogen in the hepatopancreas during Stage D, is discussed. The utilization of glycogen by the epidermis during Stages A and B, periods of inanition, is also discussed.

5. During Stages A and B alkaline phosphatase is heavily concentrated in the distal ends of the outer epidermal cells. It is observed in the integument in the region of the proximal portions of the pore canals, even before calcification begins. This is a region of high activity as deposition and hardening of the post-exuvial layers occurs. The enzyme is likewise found in the reserve cells of the subepidermal cells. In the hepatopancreas, the most marked localization of the enzyme is seen around the innumerable calcospherites in the absorption cells and in the striated border of these cells. The reserve cells of the hepatopancreas likewise show the presence of the enzyme. Functions of phosphatase in these sites are suggested.

6. Calcification begins the second day following molt and occurs thereafter simultaneously with, or immediately accompanying, the elaboration of layers of the principal zone. Calcification of the pre-exuvial layers, formed before molt, is a process accomplished long after their formation but during the same period at which the post-exuvial layers, formed after molt, are being calcified.

Calcium, heavily concentrated in the distal region of the epidermal cells, is extruded from this tissue in two distinct ways. As the post-exuvial layers are deposited, masses of calcium granules are simultaneously extruded, thus forming narrow bands paralleling the epidermis. In the pre-exuvial layers, on the other hand, calcium granules are observed in uniform vertical rows which emanate from the epidermis. These vertical rows of calcium granules correspond in location to the pore canals. Since calcification of the pre-exuvial layers occurs after their formation calcium must be transported and deposited some distance from the newly forming post-exuvial layers. This transport occurs through the pore canals, protoplasmic extensions of the epidermis, thus enabling this tissue to act at these distant sites.

7. While calcification of the integument occurs, the reserve cells in the subepidermal tissues undergo what appear to be cyclic peaks in calcium storing alternating with cyclic release to the epidermis. The reserve cells in this capacity could serve as reservoirs for providing additional calcium during periods of concentration by the epidermis. Furthermore, these interesting reserve cells, during the early postmolt period (Stages A and B), undergo at daily intervals, cyclic peaks and declines in size and abundance, changes in structural appearance, and staining properties and the storing of reserves other than calcium. The mucopolysaccharide material, either muco- or glycoprotein, in the reserve cells disappears near the latter part of Stage B. This indicates a decrease in the concentration of the material and suggests that the mucopolysaccharide stored by the reserve cells represents reserve material for the construction of the new skeleton.

The reserve cells of the hepatopancreas, like those of the integumental tissues, undergo during the early postmolt period (Stages A and B) cyclic peaks and declines in number, size and the storing and apparent release of reserves. They similarly show the presence of phosphatase, mucopolysaccharide, calcium and much lipid.

8. As calcification of the integument occurs there is a progressive decrease in number of calcospherites—spherules of calcium phosphate—present in the absorption cells of the hepatopancreas. These calcospherites, abundant preceding molt and on the first day following molt, progressively decrease in number as calcification of the new integument occurs and by the seventh day (end of Stage B) hardly a single calcospherite can be detected. The calcospherites, premolt storage depots of reserve phosphate from the old skeleton, probably represent a major source from which phosphate can be mobilized for hardening of the new skeleton during Stage B, a time at which the animals do not feed.

9. Development of the inner integument of the branchiostegites is completed in *Panulirus* during a period of three days preceding molt and two days following molt. Calcification of the inner integument, as in the pre-exuvial layers of the outer integument, occurs after its formation via the pore canals, and portions of this integument are completely calcified by the seventh day following molt (end of Stage B).

10. Calcium carbonate, the principal salt of the spiny lobster skeleton, constitutes approximately 42% of the total mineral deposited and is precipitated in the organic matrix as calcite, rather than aragonite or amorphous calcium carbonate.

11. The roles of carbonic anhydrase and citric acid in the calcification of the integument of Crustacea are discussed.

12. Continued accretionary growth of the skeleton and the hardening of it by calcification are two major tasks confronting the crustacean from Stage A to late Stage C. Although the epidermis takes the lead in the performance of these duties, the importance of the other tissues, namely the hepatopancreatic and sub-epidermal tissues, should not be under-estimated.

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DEVELOPMENTAL MODIFICATIONS IN THE SAND DOLLAR CAUSED BY COBALTOUS CHLORIDE IN COMBINATION WITH SODIUM SELENITE AND ZINC CHLORIDE [1]

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Studies on the effects of chemical agents on early developmental patterns in echinoderms have shown that similar modifications may often be caused by entirely different agents. Some groups of totally different substances will cause entodermization of the young embryo while others will produce the opposite effect (ectodermization) if concentrations and exposure periods are carefully controlled. Some will increase the area of the ventral field while others will decrease it. In recent studies (Rulon, 1952, 1953, 1955, 1956) it has been found that compounds such as sodium selenite, nickelous chloride, zinc chloride, and cobaltous chloride will all cause the loss of bilaterality and a polar elongation of the larva. Such larvae differentiate with reference to the new pattern and are very similar to each other, irrespective of the compound used.

When two unlike substances produce similar effects certain questions may be asked: (1) Do these agents affect identical reaction systems in the morphogenetic process? Or (2) are *different* loci in the reaction complex affected in such a way that the final results are the same? A definite answer to either question is difficult to make but it is believed that at least a partial answer may be suggested by the use of combinations of the different agents.

In our experiments it has been customary to submit the eggs and early embryos to a wide range of concentrations when testing the effects of any particular agent. This range usually extends from a concentration that is lethal in a few hours to one that has little or no effect. The range is commonly set up so that the succeeding steps are each one-half the concentration of the preceding (*i.e.*, *M*/100, *M*/200, *M*/400, *M*/800, *M*/1,600, *M*/3,200 . . .). When newly fertilized eggs from the same lot are distributed throughout the various concentrations, interesting comparisons may often be made. The stronger solutions commonly give proportionate inhibition—that is, all structures are grossly inhibited. With decreased concentrations the inhibition may be disproportionate (differential) in that certain processes or structures are strongly affected while others are affected slightly if at all. The differential inhibition of one structure often provides for the physiological release of another to the extent that the structure not inhibited increases in size beyond the normal.

The present work deals chiefly with combinations of agents. Since previous investigations have shown that cobaltous chloride, sodium selenite, and zinc chloride

[1] This investigation was supported by the Graduate School of Northwestern University. The writer is also much indebted to Dr. L. R. Blinks, Director of the Hopkins Marine Station, Pacific Grove, California, for many considerations.

all cause the development of sand dollar embryos that are quite similar, it seemed important to determine if these agents could replace one another in inhibitory solutions. Would the effects of these different agents be antagonistic, additive, or possibly synergistic?

Material and Methods

This work was conducted at the Hopkins Marine Station, Pacific Grove, California, during the summers of 1954–55. The adult sand dollars (*Dendraster excentricus*) were dredged from Monterey Bay and maintained in the laboratory in running sea water. Ovaries were exposed by removing the oral surfaces of the animals. The bright red eggs, exuding in droplets, were washed into finger bowls. After several washings in sea water the ova were fertilized by the addition of a few drops of sperm suspension. Only ova that were over 95 per cent fertilizable were used. All test solutions were made up in sea water and the controls and tests were always from the same batch of eggs. All eggs developed under uncrowded conditions in finger bowls, out of direct sunlight and under the moist conditions of the aquarium room where the temperature varied by no more than one degree from 18 degrees C. and smoking was not permitted.

Experimental

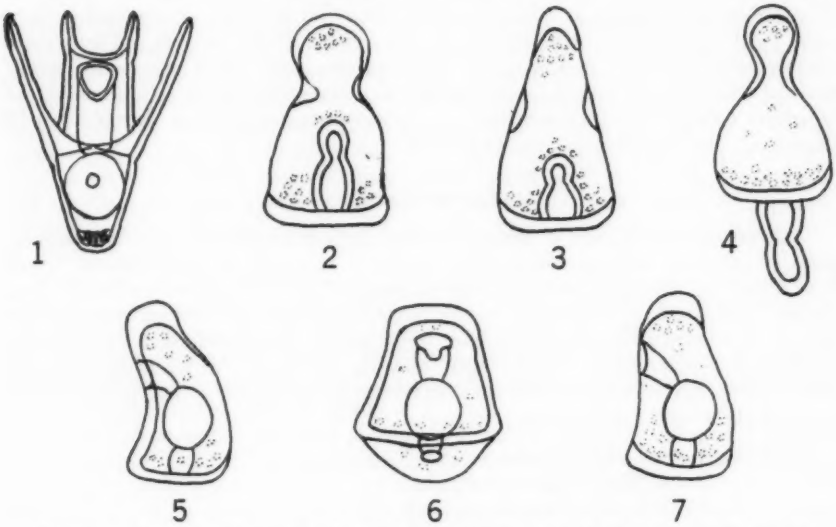
1. *Continuous exposure of newly fertilized eggs to single and combination solutions of cobaltous chloride and sodium selenite.* It would be repetitious to report here all of the modifications caused by solutions of only cobalt and selenium (see Rulon, 1952, 1956). The object of this paper is to make comparisons between the effects of solutions containing the ions singly and in combinations at critical concentrations of the range. Accordingly, only the effects of the following solutions will be discussed although additional data have been obtained from various other concentrations and combinations:

Solution 1—Sea water control
2—*M*/800 cobaltous chloride
3—*M*/1,600 cobaltous chloride
4—*M*/800 sodium selenite
5—*M*/1,600 sodium selenite
6—*M*/1,600 cobaltous chloride—*M*/1,600 sodium selenite (50 cc. of *M*/800 $CoCl_2 \cdot 6H_2O$ plus 50 cc. of *M*/800 Na_2SeO_3)
7—*M*/3,200 cobaltous chloride—*M*/3,200 sodium selenite (50 cc. of *M*/1,600 $CoCl_2 \cdot 6H_2O$ plus 50 cc. of *M*/1,600 Na_2SeO_3)

After 48 hours almost all (98 per cent) in solution 1 (control) had developed into normal free-swimming bilateral plutei with well-differentiated oral and anal arms and with full skeletal development (Fig. 1). In solution 2 all were radial in symmetry and almost all (95 per cent) showed polar elongation (Figs. 2–4). Most showed differentiation of an apical lobe (Figs. 2, 4). All had thickened basal regions and commonly a basal circle of cilia was to be seen. Exogastrulation was present in 20–30 per cent and skeletal development was inhibited in all. All were slow-moving bottom forms. In solution 3, which was one-half the strength in cobaltous chloride of solution 2, less than 10 per cent resembled the radial elongated

forms of solution 2. Instead, approximately 90 per cent were bilateral, free-swimming larvae (as Figs. 5–7). The majority of these larvae showed well-differentiated entera and stomodaea but no differentiation of skeleton or arms. There were some indications of polar elongation but they were not nearly so pronounced as in the larvae that had developed in solution 2. Approximately 20 per cent showed exogastrulation.

After 48 hours in solution 4 almost all (95 per cent) were radial forms with very little movement (Figs. 8–11). Over 75 per cent showed considerable polar elongation (Figs. 10–11) with differentiation of apical and basal lobes. No skeleton differentiated and many had basal ciliated bands. Only a few exogastrulae appeared. Approximately 75 per cent of the eggs developing in solution 5 resulted in bilateral



FIGURES 1–7. Figure 1, normal 48-hour larva. Figures 2–4, 48-hour larvae that have been exposed continuously to $M/800$ cobaltous chloride. Figures 5–7, 48-hour larvae exposed continuously to $M/1{,}600$ cobaltous chloride.

plutei with short anal arms containing skeleton and at increased angles (Figs. 12–13). Oral lobes were broad but poorly differentiated. The remainder of the larvae in solution 5 graded from slightly bilateral forms without skeleton (Fig. 14) into the polar elongated radial larvae of higher concentrations (Figs. 10–11). Only an occasional exogastrula was seen.

Almost 100 per cent of the eggs developing in solution 6 became radial or near radial larvae (Figs. 15–18). Of these, over 50 per cent showed polar elongation with oral and basal lobes (Figs. 15–17). Approximately 10 per cent were exogastrulae. In solution 7, there was only an occasional elongated radial form while approximately 90 per cent were bilateral free-swimming larvae with ventral ciliated bands but no skeleton or arms (Figs. 19–21). Approximately 10 per cent were exogastrulae but the remainder had well-differentiated entera with stomodaea.

From these experiments it was shown that when the eggs of *D. excentricus* were exposed to a combined solution of cobalt and selenite ($M/1{,}600$ $CoCl_2$–$M/1{,}600$ Na_2SeO_3) they differentiated according to a pattern of radial symmetry very much as if they had been exposed to single solutions of double strength of either agent. That is, while $M/1{,}600$ cobalt alone caused 10 per cent radial and $M/1{,}600$ selenite alone caused 25 per cent radial, together they caused 100 per cent radial (approximately 100 per cent radial forms are caused by $M/800$ of either agent). In the case of polar elongation the combined solution gave a considerably higher percentage than did the single solutions ($M/1{,}600$ $CoCl_2$ or $M/1{,}600$ Na_2SeO_3) but not as high as single solutions of double strength. Exogastrulation was highest in the cobalt,

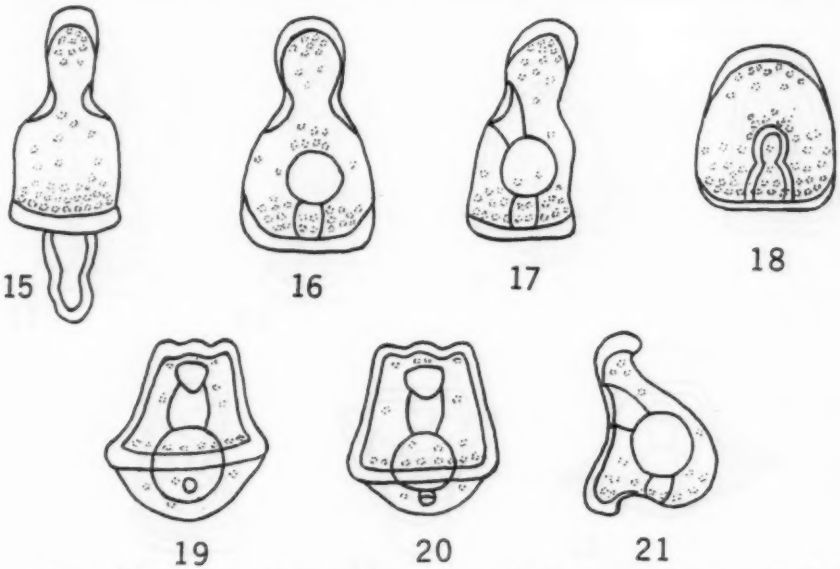
FIGURES 8–14. Figures 8–11, 48-hour larvae that have been exposed continuously to $M/800$ sodium selenite. Figures 12–14, 48-hour larvae that have been exposed continuously to $M/1{,}600$ sodium selenite.

next in the combination, and least in the selenite solutions. The data have demonstrated that the actions of these two different agents are additive in most respects in affecting developmental pattern but that there are also some effects caused by one agent but not (at least to an appreciable degree) by the other at the concentration used.

2. *Continuous exposure of newly fertilized eggs to single and combination solutions of cobaltous chloride and zinc chloride.* As in the preceding experiments, eggs from the same lot were placed in wide ranges (single and combination) of concentrations. Since the single effects have previously been reported (Rulon, 1955, 1956) only the effects of selected solutions on development shall be reported here. The solutions were as follows:

Solution 8—Sea water control
9—$M/3{,}200$ cobaltous chloride
10—$M/80{,}000$ zinc chloride
11—$M/160{,}000$ zinc chloride
12—$M/3{,}200$ cobaltous chloride—$M/160{,}000$ zinc chloride (50 cc. of $M/1{,}600$ $CoCl_2 \cdot 6H_2O$ plus 50 cc. of $M/80{,}000$ $ZnCl_2$)

After 48 hours the eggs developing in the sea water control (solution 8) were practically 100 per cent normal free-swimming bilateral plutei with good development of oral and anal arms. In solution 9 all were slightly flattened bilateral larvae



FIGURES 15–21. Figures 15–18, 48-hour larvae that have been exposed continuously to a combination solution ($M/1{,}600$ cobaltous chloride-$M/1{,}600$ sodium selenite). Figures 19–21, 48-hour larvae that have been exposed continuously to a combination solution ($M/3{,}200$ cobaltous chloride-$M/3{,}200$ sodium selenite).

which were either actively swimming or moving about near the bottom of the culture (Figs. 22–24). Approximately 20 per cent had short anal arms with skeletal spicules and 10–20 per cent had undergone exogastrulation. While these larvae were definitely in advance of those treated with cobalt solutions of twice the strength (solution 3), they still showed markedly the effects of the agent.

In solution 10 all of the larvae (Figs. 25–27) were slow-moving and radial with a large differentiated gut which commonly extended to the apical end (Fig. 25) although there were approximately 10 per cent which showed exogastrulation. These larvae had no skeleton and there was an excess of internal cells. Most showed apical thickenings or extensions although the polar elongation fell short of

FIGURES 22–33. Figures 22–24, 48-hour larvae that have been exposed continuously to *M*/3,200 cobaltous chloride. Figures 25–27, 48-hour larvae that have been exposed continuously to *M*/80,000 zinc chloride. Figures 28–30, 48-hour larvae that have been exposed continuously to *M*/160,000 zinc chloride. Figures 31–33, 48-hour larvae that have been exposed continuously to a combination solution (*M*/3,200 cobaltous chloride-*M*/160,000 zinc chloride).

that noted in certain effective solutions of cobalt and selenium. In solution 11 (which was one-half the concentration of zinc chloride as solution 10) over 90 per cent of the larvae were bilateral (Figs. 28–30) and over 50 per cent of these had short anal arms with skeleton. The remaining 10 per cent approached radial symmetry and there was approximately 5 per cent exogastrulation.

In solution 12 (combination solution) over 90 per cent were elongated radial forms with neither skeleton nor arms (Figs. 31–33). Slight bilaterality was evidenced in the remaining 10 per cent. Most had well-developed entera but 20–30 per cent had undergone exogastrulation.

These experiments show that zinc chloride causes modifications similar to those caused by cobaltous chloride but in concentrations that are 1/100th those of the latter. The addition of $M/3{,}200$ cobalt to $M/160{,}000$ zinc gives an effect which is approximately that of a single solution of zinc of twice the concentration ($M/80{,}000$) but not that of a single solution of cobalt of twice the strength ($M/1{,}600$). Instead the effect is that of a cobalt solution of four times the concentration ($M/800$).

Discussion

Previous work by the author has shown that several substances are effective in causing polar elongation and differentiation around a radial symmetry in the developing sand dollar embryo. The present data show that cobalt and selenite at the same concentrations will produce such modifications. They also show that the effects of these two different agents were additive in causing radial symmetry and almost additive in polar elongation and exogastrulation.

Other work has indicated that the radial symmetry described here is the result of a process of ventralization (see Rulon, 1949) rather than a direct inhibition of the factors which give bilaterality to the normal embryo. It has been shown (see Child, 1941) that the ventral side of the early blastula has greater indophenol oxidase (cytochrome oxidase) activity than the dorsal side, even though visible morphological differences are not apparent. Neither cobalt nor selenite appears to inhibit the activity of ventral as much as dorsal regions. The ventral area therefore spreads until it encircles the entire embryo. It is suggested that the similar effects of cobalt and selenite may be related to their known inhibitory action of thiol groups (see Rulon, 1955) and that enzymes important in symmetry relationships bear active sulfhydryl radicals. It is further suggested that these enzymes are more dorsally located at the stage of development preceding visible bilaterality. Cytochrome oxidase, whose greater activity at the ventral side has been proven, does not possess an active sulfhydryl radical (Sumner and Somers, 1953, p. 9).

Polar elongation seems to have certain factors in common with ventralization. Child (1941) also showed a polar gradient of cytochrome oxidase activity in these eggs with the highest activity at the animal pole. If it is assumed that cobalt and selenite are inhibiting activities other than cytochrome oxidase and more basally located, then it would follow that the apical end would grow and become extended at the expense of basal regions.

Zinc was highly effective in causing ventralization and polar elongation, and at a concentration far below that of the other two agents. In other words, it was more effective in inactivating, or partially inactivating, certain of the factors or processes concerned with bilaterality and polarity. It may not be unreasonable to suggest that the affinity of the thiol groups of certain enzymes for this ion may be much greater than for cobalt or selenite ions. However, the picture may be more complicated, as shown by the fact that a solution of zinc ($M/160{,}000$) which is effective in causing 10 per cent radial forms will, when administered along with $M/3{,}200$ cobalt, which causes no radial forms, cause 90 per cent radials. This would seem to suggest a synergistic action although the complexity of the phenomenon does not lend itself to an easy interpretation.

Cobalt was much more effective in the concentration used than was selenite in causing exogastrulation and skeletal inhibition. This may be because the differ-

entiation of gut and skeleton is of a more specific nature and therefore subject to more specific influences than is either ventralization or polar elongation.

Summary

1. Newly fertilized eggs of *D. excentricus* were allowed to develop in single and combined sea water solutions of cobaltous chloride, sodium selenite, and zinc chloride.

2. Combination solutions of cobalt and selenite were additive in causing the development of radial larvae and almost additive in causing polar elongation of the larvae.

3. In solutions of equivalent strength the development of exogastrulae was highest in cobalt, next in the combination, and least in the selenite solutions.

4. Solutions of zinc caused effects that were similar to those of cobalt but in concentrations that were 1/100th the latter.

5. Combination solutions of cobalt and zinc gave effects that indicated synergistic action.

6. It is suggested that the effects of cobalt, selenite, and zinc are through their reaction with the thiol groups of certain enzymes and that the greater effect of zinc is because of a greater affinity for such groupings.

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NOTES ON THE LIFE-CYCLE OF AZYGIA ACUMINATA GOLDBERGER, 1911 (AZYGIIDAE–TREMATODA)

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Studies of trematodes belonging to the genus *Azygia* Looss, 1899 from North American fresh-water fish, date from the work of Leidy (1851). He described *Distoma longum* from the stomach of *Esox ester* LeSueur, 1818 from near Cleveland, Ohio. Since that time many workers have added to knowledge of the North American species belonging to this genus. Manter (1926) gave a systematic review of the family *Azygiidae* and stated (p. 57) that "Azygia is the only genus of the family showing taxonomic confusion in its species." He further pointed out that these forms are all very muscular and highly contractile, which not only alters the general shape of the worm but also changes the relative position of such structures as the acetabulum and the reproductive organs. He also mentioned that size of the eggs and distribution of the vitellaria vary considerably within a species and cannot be relied on as taxonomic characters. Manter recognized three valid North American species: *A. acuminata* Goldberger, 1911, *A. angusticauda* (Stafford, 1904) and *A. longa* (Leidy, 1851).

Van Cleave and Mueller (1934) endorsed the action of Manter in reducing the number of species in North America and felt that *A. acuminata* should also be reduced to synonymy with *A. longa*.

Stunkard (1956) gave a chronological account of the genus *Azygia* and noted (p. 266) "—discordant observations and divergent opinions," concerning the proposed specific and generic names for members of the genus. This thorough account need not be repeated here. Stunkard recognized *A. sebago* as a distinct species and suggested that the European *A. lucii* may also be present in North America and that it possibly is distinct from *A. longa*.

None of the species have been studied to determine the extent of variation that normally occurs as the result of early development in a wide variety of paratenic hosts (small fishes and planarians) and further development in varied definitive hosts (large fishes). Until such studies are undertaken, the taxonomic picture of the group will remain confused.

It is evident, in reviewing the literature, that species proposed by various North American authors and since placed in synonymy may indeed be valid species. In such worms, which are very muscular and highly contractile, size and shape can not be relied on as taxonomic characters except in a very general way. The fact that these worms become sexually mature while relatively small and continue to grow throughout life further complicates the taxonomic picture. *A. angusticauda* is the only North American species which can be readily distinguished from the other species described from this continent. It can be separated because of the more

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posterior position of the acetabulum and the presence of the gonads in the posterior one-sixth of the body (usually within the posterior one-seventh to one-eighth of the body). The rest of the species comprise a complex composed of *A. longa, A. sebago, A. lucii* and *A. acuminata.* This complex may perhaps include as yet unrecognized species, or species which at the present time are represented as identical to *A. longa.*

The descriptions of the cercaria of *A. longa* by Sillman (1953a, 1953b), of the cercaria of *A. sebago* by Stunkard (1956), and the description of the cercaria of *A. acuminata* in the present report complete the life-histories of all of the recognized species of *Azygia* in North America with the possible exception of *A. lucii* and *A. angusticauda.* Dickerman (1937) named *Cercaria angusticauda* as a new species, but did not describe the larva.

Moreover, in discussing the cystocercous cercariae of North America Horsfall (1934) pointed out that *C. wrighti* Ward, 1916, *C. anchoroides* Ward, 1916, and *C. brookoveri* Faust, 1918 appear to be typically azygiid in morphology and should logically develop into adult azygiids. One of these cercariae may develop into *A. lucii,* but if so it is morphologically distinct from the European larva.

Szidat (1932) showed that *Cercaria mirabilis* Braun is the larva of *A. lucii* in Europe. Sillman (1953a) described the larva of *A. longa* from the snail, *Amnicola limosa,* on the basis of experimental and limited natural infections. Sillman (1953b) added additional information concerning the life-cycle of *A. longa* which was reported from both the mud pickerel, *Esox vermiculatus* and *Amia calva* in the vicinity of Ann Arbor, Michigan. Sillman also assigned worms from the bowfin, *Amia calva,* to the suppressed species, *A. acuminata.* He was unable to experimentally obtain infections of *Amnicola* from eggs of *A. acuminata.*

Stunkard (1950) identified larval distomes from the pharyngeal pockets of planarians, *Dugesia tigrinum,* as immature azygiids. Planarians serve as paratenic hosts since further development of the distomes does not occur in this host. Stunkard (1956) reported on the life-cycle of *Azygia sebago* and experimentally showed that larval stages develop in *Amnicola limosa;* further that the cystocercous cercaria is distinct from that reported by Sillman (1953a, 1953b) from the same snail host. Stunkard also reported that these cercariae were ingested by small fish (guppies and small blue gill sunfish, *Lepomis macrochirus*) and also were obtained experimentally and naturally from the pharyngeal pockets of planarians, *Dugesia tigrinum.* He stated (p. 265), "How the larvae reach the pharyngeal pockets is not clear." On the basis of known feeding habits of planarians, he correctly suggested the manner in which the distomes enter the pharyngeal pockets but did not observe it.

In preliminary observations during the present study, cystocercous cercariae belonging to both *A. sebago* from *Amnicola limosa* and *A. acuminata* from *Campeloma decisum* (Say) were placed in finger bowls with *Dugesia tigrinum.* The planarians reacted to close proximity of both species of larvae by raising the anterior end of the body and, when the swimming cercaria came in contact with the under surface of the planarian, actively enclosing the cercaria against the glass container. The pharynx was then extruded and the cercaria was sucked into the protruded pharynx. Usually the tail entered first, always so in cercaria of *A. acuminata.* The distome portion of *A. sebago* was sucked into the pharynx where it was gradually divested of the tail and the distome became active and eventually

PLATE I

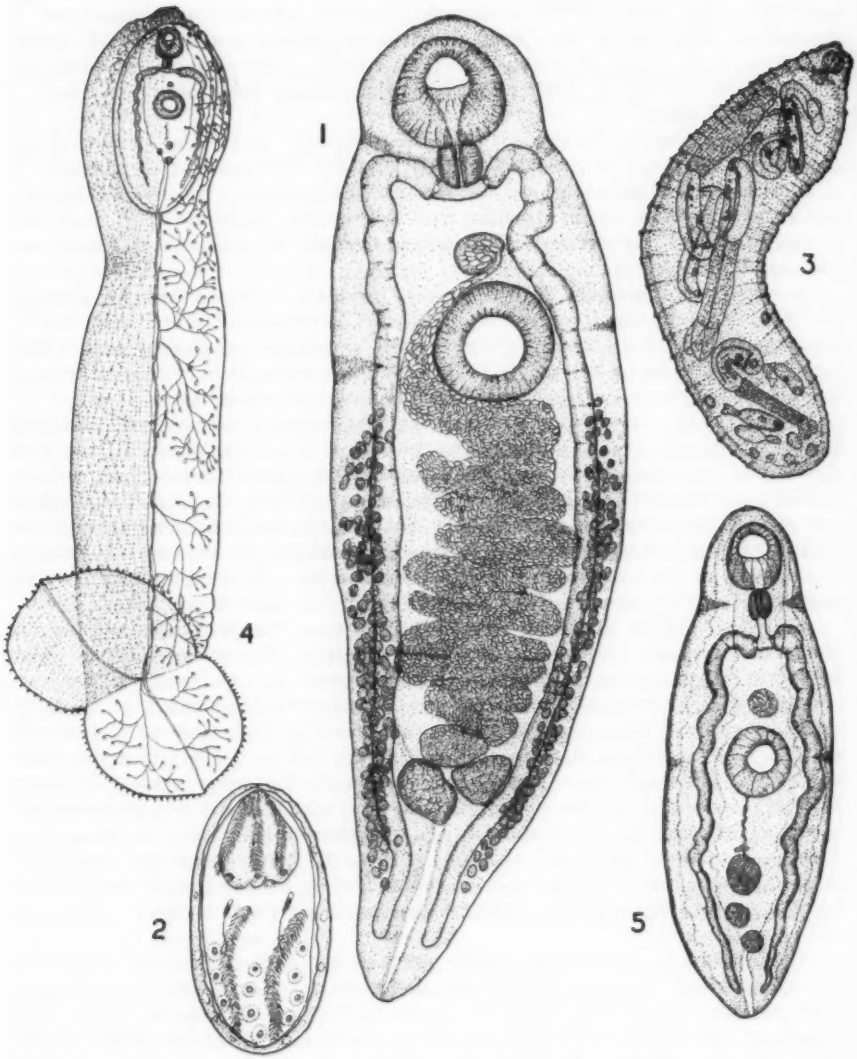

FIGURE 1. *Azygia acuminata*, adult (5.1 mm. in length), ventral view, collected from *Ameiurus nebulosus*.

FIGURE 2. *Azygia acuminata*, miracidium in egg (0.064 mm. in length), from sketches made of living larvae.

FIGURE 3. *Azygia acuminata*, redia (2.52 mm. in length), showing recognizable pharynx, annulations of the body and developing cercariae.

crawled out of the pharynx into the pharyngeal pocket. Up to six *A. sebago* larval distomes were seen to thus enter a single planarian. The larvae of *A. acuminata* were also enfolded by the planarians. The tail was sucked in but the larger size of the distome prevented both its entrance into the pharynx as well as into the aperture to the pharyngeal pocket. Occasionally a distome would be sucked part way into the pharynx and would remain so for several hours before finally becoming detached. Observations indicated that *D. tigrinum* could and probably does serve as a normal paratenic host for *A. sebago* but not for *A. acuminata*.

The cercaria of *A. acuminata* was found in *Campeloma decisum* obtained from the Santuit River, a small stream near the settlement of Santuit, Cape Cod, Mass. Small fishes, sticklebacks, *Eucalia inconstans* (Kirtland), *Fundulus heteroclitus*, the trout, *Salvelinus fontinalis* (Mitchill), and small eels, *Anguilla rostrata* LeSueur were obtained from streams known to be free of azygiid infections and were utilized as paratenic hosts. Specimens of each lot were examined and found to be free of azygiids and the remainder used, after infection, in feeding the definitive host fishes. These were found to be bull-heads, *Ameiurus nebulosus* LeSueur; blue gill sunfish, *Lepomis macrochirus* Raf.; chain pickerel, *Esox niger* LeSueur; and yellow perch, *Perca flavescens* (Mitchill). Yellow perch do not occur in this stream. These hosts were collected from a small pond known to be free of azygiids and were experimentally infected.

Large numbers of snails, *Campeloma decisum*, were kept in shallow, well aerated aquaria. In some instances liberated cercariae were pipetted from the aquarium and fed to small fish, and in other cases small fish were kept in the same aquarium. Heavy infections resulted in both cases since the swimming cercariae were readily ingested by the fish.

The young distomes differed in their relationship to the different species of small fish. Young trout and *Fundulus heteroclitus* retained an infection for only six to seven days. During this time the worms became less active and were found further and further posterior in the intestine of the fish. Sticklebacks and eels retained most of their infection in the stomach region. Worms removed from the latter species of fish were more active and their caeca were crowded with ingested material. Sticklebacks retained up to ten worms in the anterior portion of the intestine for as long as two weeks, whereas eels were found to have the stomach crowded with as many as twenty-four distomes.

Small fish were ingested by the larger fish used in the feeding experiments. The pectoral and dorsal spines of the sticklebacks were clipped before placing them in the aquarium with the definitive hosts.

Stages in the Life-Cycle

Adult (Fig. 1)

The original description of *A. acuminata* by Goldberger (1911) emphasized taxonomically-unimportant points such as zigzag caeca, rounded cephalic and

FIGURE 4. *Azygia acuminata* cercaria, naturally emerged, from sketches of living specimen (4.20 mm. in length). Flame cell pattern of the tail, transposed from sketches made from other cercariae, shown on right side of tail. Detail of musculature shown on the left, omitted on the right side of the tail.

FIGURE 5. *Azygia acuminata*, juvenile worm (1.18 mm. in length), experimental infection, from stomach of small eel, *Anguilla rostrata*.

pointed caudal ends, uninterrupted vitellaria and constricted neck region in his specific diagnosis.

Manter (1926) studied Goldberger's material, also material identified by A. R. Cooper, and specimens from H. B. Ward's collection. He suggested that certain similarities in the material showed *A. acuminata* to be a valid species. Manter stated (p. 61), "The most distinguishing specific characters were found to be: relatively wide body, anterior extent of the vitellaria, egg size, and poorly developed condition of the internal parenchyma muscles. It should be realized that the nature of all of these features is of somewhat precarious standing in this group. Probably no one of them, unless very marked, would justify recognition of a separate species. Only because of the general association of all of these characters can the forms be separated from the other common American species."

Van Cleave and Mueller (1934) regarded *A. acuminata* and *A. bulbosa* of Goldberger (1911) as synonyms, and since Manter had regarded *A. bulbosa* as a synonym of *A. longa*, they added *A. acuminata* to the long list of synonyms of *A. longa*. They pointed out that while the anterior extent of vitellaria was an important taxonomic character in the genus, the comparative width, constriction of the neck, and insignificant differences in egg size did not justify maintaining *A. acuminata* as a valid species. They stated that the less highly developed condition of the internal muscles was a dubious character. On the basis of the present study it appears evident that Van Cleave and Mueller were not justified in reducing *A. acuminata* to synonymy with *A. longa;* that *A. bulbosa* should be considered synonymous with *A. acuminata;* and the species should be maintained in the genus *Azygia*.

Emended description of the adult

Characters of the genus, body, 4–10 mm. long, width usually less than one-fifth of the length. Body characteristically slightly constricted anterior to the acetabulum. Position of constriction variable depending on state of contraction of the worm. Acetabulum one-fourth to one-third total length from anterior end of body. Genital pore and cirrus sac median in position immediately cephalad of acetabulum. Oral sucker 0.25–0.90 mm. in diameter, acetabulum slightly larger, 0.28–0.95 mm. in diameter. Gonads contiguous. Posterior testis larger than anterior testis. Vitellaria extra-caecal, extending from level of posterior margin of acetabulum to level midway between posterior testis and caudal end of body. Eggs consistently larger than in other described azygiid species, 0.64–0.69 × 0.30–0.34 mm.

Host: *Amia calva, Lepomis macrochirus,* and *Ameiurus nebulosus.*

Habitat: Stomach.

Localities: Illinois, Michigan, and Cape Cod, Massachusetts.

Miracidium

Miracidia representing all genera of the family Azygiidae have been described except for the genus *Leuceruthrus*. Azygiid miracidia lack cilia and are provided with bristle plates or plaques. The miracidium of *A. acuminata* is morphologically similar to the miracidium of *A. sebago* described by Stunkard (1956), to that of *Proterometra macrostoma* as reported by Hussey (1945), and to the earlier descriptions of that of *A. longa* by Schauinsland (1883) and Looss (1894).

Eggs must be ingested by the proper snail hosts before hatching will occur.

Attempts to hatch the eggs by placing them with material from the digestive tract of large *Campeloma decisum* were unsuccessful.

Miracidia were studied both alive in the egg (Fig. 2) and in stained sections of gravid worms. The miracidium almost completely fills the egg-shell. Radiating from the anterior end are five plaques bearing fine bristles arranged in chevron fashion with the apices anterior in position. These plaques extend posteriad about one-third of the length of the larva. A short distance from the posterior end of the miracidium, four other bristle plaques extend anteriad with a tendency to spiral, which may be due to movements of the larva.

Internally, occupying almost the anterior one-third of the miradicium, are four unicellular gland-like structures, the so-called primitive gut of earlier authors, which Stunkard considered probably secrete substances which aid in penetration. Wootton (1957) demonstrated that in *Allocreadium alloneotenicum* this group of glands does aid in penetration and that the penetration glands of earlier authors serve in forming the cuticle of the sporocyst. Attempts, in crushed snails, to observe the action of the anterior gland in the miracidium of *A. acuminata* were unsuccessful. It is possible since ciliary plates are lacking in azygiid miracidia that the miracidial covering serves also as the covering of the sporocyst, thus explaining the absence of cuticle-forming glands. Up to twelve germinal cells are visible in the posterior two-thirds of the miracidium. Paired flame cells lie near the middle of the body, one on each side, each with a duct leading caudad. The ducts could not be clearly traced to their pores.

Sporocyst

Young snails were removed directly from the uterus of an uninfected female *Campeloma decisum*. The young snails readily fed in a layer of clean sand. Eggs of *A. acuminata*, each containing an active miracidium, were added to the sand in which the snails were feeding. Snails were dissected at the end of one, two, and three weeks but no infection was found. It is probable that the snails must be larger before infection will occur.

Rediae

Infections with larvae of *A. acuminata* were present only in females of *Campeloma decisum*. Infections of male *Campeloma* were never observed. The redial stages were present, usually with unencysted metacercariae identified as *Leucochloridiomorpha constantiae* (Mueller), in the uterus of the snail. The loci of infection did not extend into the digestive gland, the usual site of infection for larval trematodes. Up to eighty rediae were dissected from the uterus of a single snail. These, plus almost as many metacercariae of *L. constantiae*, completely filled the uterus. Embryo snails, which were usually found in the uterus of uninfected females, were absent in infected snails. In a few instances partially empty embryo snail shells were found in the uterus, but it was evident that infection with larval *A. acuminata* adversely affected normal development of the young snails. The redial stages undoubtedly derived their nourishment from the developing embryo snails within the uterus. Young snails develop normally when associated only with metacercariae of *L. constantiae*. Thus, infection of the snail with larvae of

A. acuminata caused the degeneration of the young snails. Rediae (Fig. 3) when fixed averaged 2.15 × 0.71 mm.

The vermiform rediae are very active and are capable of extending to a length of over 5 mm. When contracted, the external body wall is formed into regularly spaced annular rings, giving it a wrinkled appearance. Even when fully extended, these rings persist as fine annular projections. The body wall is 0.043 mm. in thickness in sectioned material. The small pharynx is not easily observable in stained whole mounts, but is visible in sections as a rudimentary structure (0.11 mm. in diameter) with a well defined lumen. No recognizable birth pore can be seen, either in sectioned materials or in stained whole mounts or rediae. It is probable that cercariae escape through the pharynx.

Up to twelve recognizable cercariae are present in each redia, with seven to ten being the usual number. Other developing cercariae are present as germ-balls, and a maximum number of twelve developing cercariae present with six germ-balls was observed. The redial stage is very similar to that reported by Szidat (1932) for *A. lucii* and by Stunkard (1956) for *A. sebago,* varying only in size and in number of developing cercariae.

Cercariae

The development of the cercaria is typical of that reported for cystocercous larvae. The tail becomes evident in development when the larva reaches a length of only about 0.1 mm. When the larva reaches a total length of 0.5–0.6 mm. the furcal buds appear as oval projections. At this stage the suckers are recognizable, and the primordia of one testis and the cirrus sac are also visible as deeper staining areas. The tail increases more rapidly than the distome during further development. The largest cercaria observed within a redia as a stained whole mount measured 2.22 mm. in total length. The distome measured 0.74 × 0.37 mm.

Cercariae appear to undergo additional development in the uterus of the snail before escaping from the female genital pore. After emergence, they are quiescent for a brief period before actively swimming in a typical cystocercous fashion. Cercariae normally emerge between 12:00 P.M. and 4:00 A.M. Standard Time. Limited numbers escape during daylight hours. It appears, however, that the majority emerge during hours of darkness and are either ingested by small fish at this time or in the early hours of daylight. They live for only ten to twelve hours, becoming less active as they age. Cercariae while still within the uterus of the snail do not have the distome portion enclosed by the tail. Upon coming into contact with the water, the anterior tail bulb absorbs water, expands rapidly anteriorly thus enclosing the distome, as has been described for other cyctocercous cercariae.

Mature, normally liberated cercariae (Fig. 4) measure 3.21–4.69 mm. (averaged 4.22 mm.) in total length when infected snails are first brought into the laboratory. The size of the cercariae gradually decreases in snails kept in captivity, undoubtedly a result of deficient nutrition of the hosts. The tail stem is round in cross-section at the bulb-like anterior end enclosing the distome. This portion measures 0.69–0.96 mm. in diameter (average 0.78 mm.). Just posterior to the more or less rigid anterior bulb enclosing the distome, the tail decreases slightly to a diameter of 0.52–0.82 mm. (average 0.66 mm.). From the constricted neck-portion the tail gradually flattens and widens to a width of 0.62–1.11 mm. (average 0.84 mm.) and then tapers gradually to an average width of 0.79 mm. just anterior to the furcal branches.

The furci are broadly lobed structures, 1.11–1.28 mm. in length (average 1.21 mm.) and 0.89–1.09 mm. in width (average 0.94 mm.). Each furca has a terminal papilla on which the excretory pore opens and small regularly arranged scale-like marginal protuberances. The tail of the cercaria is colorless, slightly opaque and devoid of protuberances, spines and mammulations characteristic of *C. mirabilis* Braun, 1891, *C. macrostoma* Faust, 1918, *C. splendens* Szidat, 1932, *C. anchoroides* Ward, 1916, and *C. sebago* Stunkard, 1956. The cercaria of *A. acuminata* differs in size and in the proportionate size of the distome when compared with the tail length from other cystocercous cercariae which characteristically do not possess papillae. It differs from *A. hodgesiana* Smith, 1932 since the genital organs are not functional as they are in the latter; from *A. stephanocauda* Faust, 1921 in size and shape of the tail; from *C. wrighti* Ward, 1916 in size; and from *C. pekinensis* Faust, 1921 in proportionate size of the distome.

The cercaria of *A. acuminata* is most like *C. brookoveri* Faust, 1918 and *C. anchoroides* Ward, 1916, but is over twice as large. *C. brookoveri* was originally described from crushed *Campeloma* sp. and the free-swimming larva was rediscovered by Dickerman (1937) from the same snails. Unfortunately Dickerman did not further describe the species. *C. anchoroides* was collected only in plankton tows from Lake Erie. The size and obvious similarities in structure of the two forms, as well as geographic proximity of the type localities, caused Horsfall (1934) to think that they will prove to be synonymous when the life-cycles are known.

The enclosed distome measures 0.66–0.79 × 0.37–0.47 mm. in living material. It usually lies with the oral sucker at the anterior end of the tail-bulb. It is flattened and varies as to its orientation to the width of the tail, sometimes being at right angles and at others with its width the same as the width of the tail. The excretory system is continuous with that of the tail, extending down the tail as a common excretory canal bifurcating at the bases of the furci and opening at the small points of the furci.

The structure of the larva when forced from the tail-bulb is typically azygiid. The preacetabular region bears many papillae which decrease in size and number, and are absent behind the mid-acetabular region. Living specimens, flattened slightly under a cover glass, measure from 0.98–1.38 mm. (average 1.18 mm.) in length and from 0.37–0.54 mm. in width (average 0.48 mm.). The oral sucker varies from 0.22–0.25 mm. in length and from 0.20–0.23 mm. in width. It is subterminal, opening ventrally. The pharynx measures 0.090–0.098 mm. in length and 0.49–0.61 mm. in width. The acetabulum varies from 0.22–0.25 mm. in length and from 0.25–0.29 mm. in width. It is about midlength in the body. The digestive caeca are filled with opaque material and extend almost to the posterior end of the larva. The excretory bladder extends anteriorly to the region just posterior to the testes where it branches into two main collecting ducts. These extend median to the caeca, crossing laterad as the caeca turn mediad to join the pharynx. After crossing under the caeca, the ducts pass laterally and antero-laterally to the oral sucker, continuing almost to the anterior end of the body, but they do not join. Anterio-lateral to the oral sucker each duct doubles backward and extends posteriad, lateral to the caeca, giving off eleven branches.

The first branch is located lateral to the oral sucker, the second at the level of the pharynx, the third and fourth anterior to the acetabulum, the fifth at the anterior edge of the acetabulum, the sixth lateral to the acetabulum, the seventh and eighth

are close together just behind the acetabulum, the ninth and tenth are about equally spaced in the intervening region, while the last branch continues to the posterior end of the body lateral to the excretory bladder. Each branch divides three times in a dichotomous fashion, thus forming two primary, four secondary, and eight tertiary branches. Each tertiary branch drains four flame cells. Thus the flame cell formula is 2 (11 × 32) or 704 flame cells.

The number and arrangement of the flame cells is in agreement with those reported by Looss (1894) for *Azygia terreticolla* (= *A. lucii*) and by Stunkard (1956) for *A. sebago*. While the numbers of branches and flame cells agree with these earlier descriptions, the positions of the branches are different in *A. acuminata* due to the relatively more posterior position of the acetabulum.

The excretory system of the tail is equally complex (Fig. 4). In addition to the common excretory canal extending down the center of the tail and bifurcating into each furca, two paired accessory canals paralleling the main canal were observed. One duct and its branches drained the right side of the tail and the right furca, and the other the left side and the left furca. Each duct collected from five branches but dichotomous bifurcation of the ducts was not as clearly evident as in the distome portion.

Each of the five branches, however, did drain from 32 flame cells, arranged in groups of fours. The first branch turned anteriad from just caudad of the distome, draining the enclosing bulb area, the second and third branches joined the collecting duct close together in about the middle of the tail, the second turning anteriad and draining that area, the third posteriad collecting from the third quarter of the tail. The fourth branch joined the collecting ducts about three-fourths the length of the tail and drained the final quarter of the tail. From the fourth branch, the duct extended into a furca draining from 32 flame cells. The formula for the tail is thus 2 (5 × 32) or 320 and the entire cercaria has a formula of 2 (16 × 32) or 1,024 flame cells.

The connection of the accessory ducts of the tail to the rest of the excretory system was not resolved. The dense protoplasm at the tip of the tail in immature cercariae freed from rediae and the congested area at the base of distomes enclosed in the tail of normally liberated cercariae made observations impossible. These ducts are 0.011 mm. in diameter compared to the common duct which is 0.055 mm. in diameter. Faust (1921) reported that in *C. pekinensis*, the tail had only 32 flame cells and connected to the excretory system of the distome as the eighth branch. He further reported only seven branches in the distome portion of *C. pekinensis*. The flame cell pattern of this form should be examined in the light of the observations of the cercariae of *A. lucii* Looss (1894), *A. sebago* Stunkard 1956, and the present observations of *A. acuminata*, since *C. pekinensis* would appear to also develop into an azygiid.

Young worms

Worms increased very little in size and did not undergo further development while in the stomach of small fishes. A young distome (Fig. 5) from the stomach of a small eel differed from one newly forced from the cercarial tail only in the size of the caeca. In worms taken from paratenic hosts, the caeca were enlarged with food particles. No measureable differences in worms from various hosts

were found. Worms from the sticklebacks and young eels were more active and appeared healthier than worms from other small fish.

Manter's synopsis and key to the genus *Azygia* can be revised to include *A. sebago* and the cercaria of each species can be noted as follows:

KEY TO THE SPECIES OF AZYGIA FROM NORTH AMERICA

1 (2) Vitellaria not extending appreciably posterior to the last testis. Length 6–54 mm. (*C. mirabilis* Braun, 1891) ..... *A. lucii* (Mueller)
2 (1) Vitellaria extending posteriad at least half the distance between posterior testis and end of body 3
3 (4) Acetabulum near middle of body, gonads in posterior one-sixth of body (*C. angusticauda* Dickerman, 1937) ..... *A. angusticauda* (Stafford, 1904)
4 (3) Acetabulum within anterior one-third of body, gonads more anterior 5
5 (6) Body width usually one-fifth the total length, vitellaria extending posteriad from close behind acetabulum, internal parenchyma muscles relatively weak. Eggs 0.064–0.069 × 0.30–0.34 mm. (*Cercaria acuminata*, present paper) ..... *A. acuminata* Goldberger, 1911
6 (7) Body width proportionately less than one-fifth the length, vitellaria begin some distance posterior to acetabulum, internal parenchyma muscles strongly developed, eggs variable in size but smaller than *A. acuminata* 7
7 (8) Body length not over 15 mm., usually smaller, body robust in appearance (*Cercaria sebago* Stunkard, 1956) ..... *A. sebago* Ward, 1910
8 (7) Body often extremely elongate, vitellaria beginning proportionally more posteriorly (*Cercaria longa* Sillman, 1953a) ..... *A. longa* (Leidy, 1851).

The writer wishes to express his appreciation to the Director of the Marine Biological Laboratory, Woods Hole, Mass., for the use of facilities. Further, he is particularly grateful for the helpful suggestions offered by H. W. Stunkard throughout this investigation and for his criticism of the manuscript.

Summary

1. Stages in the life-cycle of *Azygia acuminata* are described and figured. Cystocercous cercariae develop from rediae in the snail, *Campeloma decisum*. The cercaria is morphologically distinct from other described cystocercous cercariae. Rediae are similar to the same stage described for other members of the genus, but are unique since they develop in the uterus of female *Campeloma decisum*.

2. The excretory system of the cercaria is complex, showing a formula of 2 (11 × 32) or 704 flame cells for the distome portion and 2 (5 × 32) or 320 flame cells in the tail. The excretory formula of the cercariae is thus 2 (16 × 32) or 1,024 flame cells.

3. Attempts to experimentally infect small snails, taken from the uterus of a *Campeloma decisum*, by feeding them eggs of *A. acuminata* were not successful.

4. Various small fishes were utilized as paratenic hosts by the young distomes. Infections in sticklebacks, *Eucalia inconstans*, and small eels, *Anguilla rostrata*, resulted in more active and vigorous worms than did infections from other paratenic hosts.

5. The variation that normally occurs in members of the genus *Azygia* due to development in a wide variety of hosts is not known. Consequently diagnostic characters of mature worms can not be relied on to distinguish species. On the basis of this report the suppressed species *A. acuminata* is regarded as a valid species and should be retained in the genus *Azygia*.

6. *A. acuminata*, previously reported only from *Amia calva*, was found occur-

ring naturally in bullheads, *Ameiurus nebulosus,* blue gill sunfish, *Lepomis macrochirus,* and chain pickerel, *Esox niger,* from Santuit River, Barnstable County, Cape Cod, Massachussetts. Experimental infections were also obtained in these fishes and in the yellow perch, *Perca flavescens.*

7. A revised key for the genus *Azygia* is presented, listing the recognized species and the described cercarial stages.

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Volume 113 Number 3

UNIVERSITY OF MICHIGAN
JAN 27 1958
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DECEMBER, 1957

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

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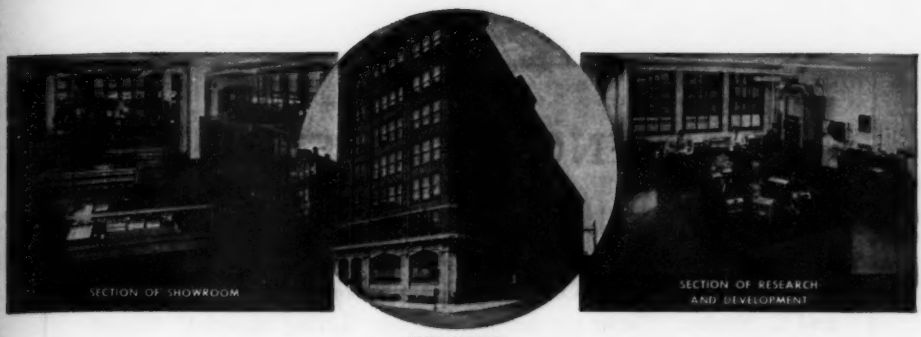
THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 1 and September 1, and to Dr. Donald P. Costello, P. O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa. under the Act of August 24, 1912.



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